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Research

Rapid report

The flower of *Hibiscus trionum* is both visibly and measurably iridescent

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Summary

- Living organisms can use minute structures to manipulate the reflection of light and display colours based on interference. There has been debate in recent literature over whether the diffractive optical effects produced by epoxy replicas of petals with folded cuticles persist and induce iridescence in the original flowers when the effects of petal pigment and illumination are taken into account.
- We explored the optical properties of the petal of *Hibiscus trionum* by macro-imaging, scanning and transmission electron microscopy, and visible and ultraviolet (UV) angle-resolved spectroscopy of the petal.
- The flower of *Hibiscus trionum* is visibly iridescent, and the iridescence can be captured photographically. The iridescence derives from a diffraction grating generated by folds of the cuticle. The iridescence of the petal can be quantitatively characterized by spectrometric measurements with several square-millimetres of sample area illuminated.
- The flower of *Hibiscus trionum* has the potential to interact with its pollinators (honeybees, other bees, butterflies and flies) through iridescent signals produced by its cuticular diffraction grating.

Introduction

In 2009 Whitney *et al.* reported for the first time that the petal epidermis of certain plant species is coated in a cuticle that is, buckled or folded in a sufficiently regular nanoscale pattern to produce diffractive optical effects. The authors measured the iridescence produced by epoxy replicas of petal surfaces, including *Hibiscus trionum* (Venice mallow). *Hibiscus trionum* is thought to be primarily pollinated by honeybees and other bees; butterflies and flies also visit the flowers (Seed *et al.*, 2006). The authors also demonstrated that foraging bumblebees could be trained to identify iridescent replicas of such striated petals as rewarding and to distinguish them from otherwise identical noniridescent replicas of smooth petals. However, they did not report direct measurements of the optical properties of the petals themselves. Since most flower petals contain pigments, it is to be expected that the overall optical properties of a petal with a folded cuticle will result from a combination of the absorption of light by pigments and optical effects stemming from surface structures. The tulip cultivar 'Queen of the Night' is an interesting example of how the response of the pigment is coupled to the photonic response of the regularly folded cuticle of the tepal (Vignolini *et al.*, 2013). Other recent analyses of the optical properties of flowers without folded cuticles, namely the highly reflective buttercup, *Ranunculus acris*, and the glossy blue speculum of the mirror orchid, *Ophrys speculum*, have confirmed that optical response is a consequence of combining pigments and structural effects (Vignolini *et al.*, 2012a,b).

In a recent paper, van der Kooi *et al.* (2014) set out to determine the contribution of cuticular folding to petal optical response. The authors measured the optical properties of the flowers of four different species, three of which had geometries that contribute to light scattering on the microscale, thereby rendering any nanoscale iridescent effects irrelevant by cancelling them out. However, they also described an optical analysis of the flower of *Hibiscus trionum*, one of the species originally described as iridescent by Whitney *et al.*

(2009). Van der Kooi *et al.* (2014) were able to replicate the observation that epoxy replicas of the *H. trionum* petal were iridescent, and were also able to record iridescent effects on the petals themselves when illuminated with a very fine spot (of 13 μm diameter). However, when optical measurements were taken using illumination over a larger spot (140 μm), the diffractive effects were blurred and the petal was not recorded as iridescent. The authors therefore concluded that pigmentary rather than structural coloration determines floral optical appearance, and that a role for cuticular folding and iridescence in floral signalling is untenable.

In this rapid report we describe our own analyses of the optical properties of *Hibiscus trionum*. We demonstrate that the flower is visibly iridescent under daylight illumination, and report optical measurements which show that it is possible to measure the diffraction effects produced by the regular cuticle folding over spot sizes in the millimetre range. We therefore conclude that a role for cuticular folding and iridescence in floral signalling is highly plausible, although its distribution and significance remain to be explored.

Materials and Methods

Plant material

Seeds of *Hibiscus trionum* L. were obtained from Chiltern seeds (http://www.chilternseeds.co.uk). Plants were grown under glasshouse conditions at 23°C in Levingtons (UK) compost. During the growth period, plants received supplemental lighting from Osram 400 W high-pressure sodium lamps (Osram, München, Germany) on a 16 h : 8 h, light : dark photoperiod.

Microscopy

The surface of freshly collected petals was imaged using cryogenic scanning electron microscopy (cryo-SEM S-4700; Hitachi, Schaumburg, IL, USA). Thus, 10 mm^2 of petal tissue (corresponding to either the white or the purple region of the *Hibiscus trionum* petal) was mounted on a stage, fixed by cooling in nitrogen slush, before being sputter-coated with gold in the antechamber of the scanning electron microscope. The samples were then introduced into the main chamber to be imaged.

For transmission electron microscopy (TEM) examination a 2 mm^2 area of petal tissue was dissected using a mounted needle, fixed in 2.5% glutaraldehyde in phosphate buffer at pH 7.4, and stored in 70% ethanol until needed. Samples were then stained in 1% osmium tetroxide solution and passed through an ethanol and resin series before being polymerized for 18 h under vacuum. Semi-thin sections (0.5–2 μm) and ultra-thin sections (50–90 nm) were cut using a Jung Ultracut microtome (Reichert; Depew, NY, USA). The semi-thin sections were mounted on glass slides and stained with toluidine blue in phosphate buffer, before being examined under a light microscope. The ultra-thin sections were placed on copper mesh grids before being examined using a Hitachi H-7650 transmission electron microscope.

Optical analysis

In order to characterize the iridescence of the petal quantitatively, we used an optical goniometer (Vignolini *et al.*, 2013). In particular, we performed angular resolved measurements in scattering configuration. In this experiment, a light source from a combined deuterium halogen lamp (DH-2000 Deuterium Tungsten Halogen Light Sources; Ocean Optics, Dunedin, FL, USA) was collimated to form a 1 mm wide parallel incident beam (divergence of *c.* 5°) that illuminates the sample at a fixed angle. The scattered light was detected for different angles with an angular resolution of 2° and coupled into an optical fibre connected to the spectrometer (QE65000 Ocean Optics, 200–950 nm). All the spectra were normalized to a white Lambertian reflectance standard (Spectralon, > 99% reflectance).

Results

The flower of *Hibiscus trionum* is visibly iridescent

The five petals of *Hibiscus trionum* are each pigmented dark red/purple with anthocyanin at the proximal portion and are white towards the distal portion (Fig. 1a,b). The white section of the petal is not iridescent and has an epidermal surface comprising smooth conical-papillate light-scattering cells (Fig. 1c). However, in the red region of the petal the epidermal cells are flat and covered with a heavily striated cuticle (Fig. 1d,e). The red region is visibly iridescent under daylight illumination (no flash), as can be seen in Fig. 1(a,b). The iridescent effect can also be observed when petals are photographed at a series of angles (Fig. 1f–h). As the angle of the petals (or the angle of observation) shifts, different colours (blue-green-gold) appear to overlay the red pigmentation. The intensity of the iridescence effect and the region where it is visible on the red area vary as the angle of observation changes (Fig. 1f–h). This phenomenon, that is, the display of different colours at different angles of observation, is called iridescence.

The *Hibiscus trionum* petal is measurably iridescent when illuminated with white light over areas of several square millimetres

To characterize quantitatively the iridescence, we performed scattering measurements of fresh petals as described in the Materials and Methods section. Since the flower is open only for a few hours, it is important to perform the optical measurements in this specific interval of time. Moreover, the spectroscopic measurements need to be performed as soon as the petal is detached from the flower, because the epidermis degrades quickly (2 h maximum). We also observed that, when tissues have been kept at 4°C, the degradation time was significantly shortened and therefore we were not able to measure iridescence.

To capture in a measurement the iridescent behaviour that is clearly visible to the naked eye under ambient illumination (Fig. 1a–b,f–h), it is necessary to take particular care when fixing the sample into the sample holder, as the petal is curved (Fig. 1). To measure the optical response of a diffracting object it is extremely

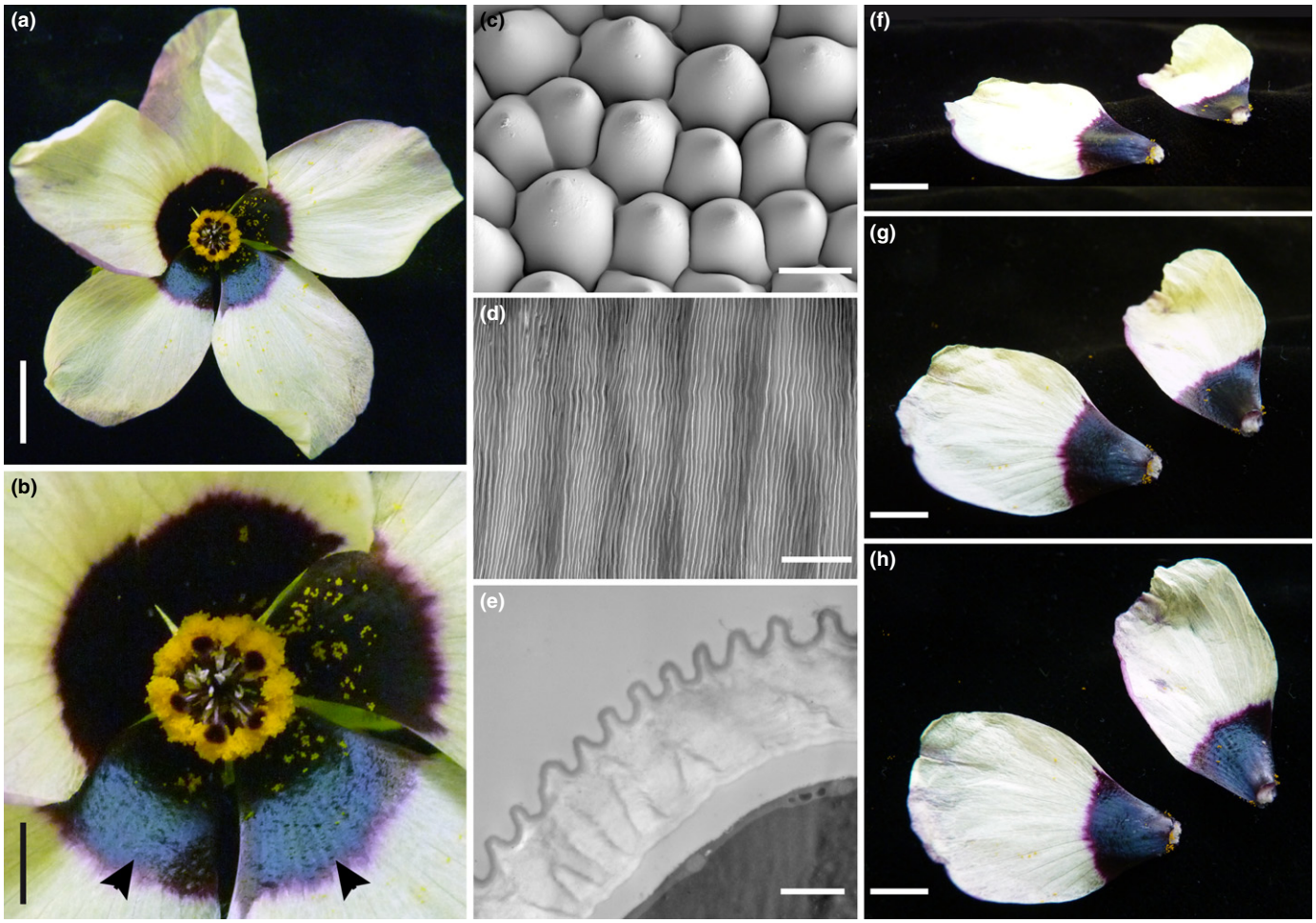

Fig. 1 Iridescence on the petal of *Hibiscus trionum*. (a) Open flower of *H. trionum* photographed in daylight illumination. The perianth shows a 'bulls-eye' pattern with a white distal region and a proximal purple area that surrounds the central reproductive organs. (b) Close-up view of the flower centre. The purple region is iridescent, as a metallic blue-green-gold sheen can been seen on this part of the petal (arrowheads). The yellow anthers have already shed some pollen onto the perianth. (c) The cells in the epidermis of the white region are conical with a smooth cuticle that does not produce any iridescence. (d) The epidermal cells in the purple region are flat and covered with a striated cuticle. (e) TEM analysis of the epidermis in the purple area of the petal reveals that the striations are part of the cuticle and constitute a diffraction grating that produces the iridescent effect. The average distance between the striations is 1.3 ± 0.3 μm. (f–h) Observation of *H. trionum* petals from three different angles (≈ 30°, 60° and 90°, respectively) shows the variation in hue, colour intensity and position on the purple surface as the observation angle varies. Bars: (a) 2 cm; (b, f–h) 1 cm; (c, d) 20 μm; (e) 2 μm.

important to mount the investigated area of sample as flat as possible in order to properly define an angle of incidence and collection and correlate the optical response to the anatomy of the petal. We fixed the petal into the sample holder using double-sided tape, without pressing the area that we wanted to measure.

Since an optical goniometer gives us access to scattered light in one dimension only, it is crucial to identify the direction of the striations on the petal before performing the measurement. This procedure can be achieved by using a standard optical microscope; the direction of the striations on the petal (already fixed to the sample holder) can be visualized with a ×20 or a ×50 objective. Once the direction of the striations had been determined, we mounted the sample holder in position in one of two configurations: (1) striations perpendicular to the plane where the detector is rotated or (2) striations parallel to the plane where the detector is rotated. A schematic representation of the setup in configuration (1) is depicted in Fig. 2(a). The sample is illuminated at an angle of incidence $\theta_i = 75°$ with respect to the sample plane, with collimated light from the lamp illuminating an area > 1 mm^2. The scattered light from the sample is collected for different angles of detection ($\theta_D = 15°, 25°, 45°, 75°$) and the corresponding spectra are shown in Fig. 2(b), clearly indicating the change of colour as a function of angle of detection (iridescence). The graph in Fig. 2(c) shows more spectra in a two-dimensional plot, where the intensity of the reflected light from the sample is shown in a rainbow colour-scale for different angles of collection. In particular, angle $\theta_D = 0°$ corresponds to the plane of the sample, while $\theta_D = 90°$ corresponds to the normal vector with respect to the sample plane. When the striations are perpendicular to the collection plane of the detector, we observed the specular reflected signal *c.* $\theta_D = 75°$ (black dotted line). In the specular reflection direction the spectral response is mainly above 700 nm, because for lower wavelengths the absorption from the pigment sets in. For angle of collection between 20° and 40° we can observe the contribution of the striations (Fig. 2c).

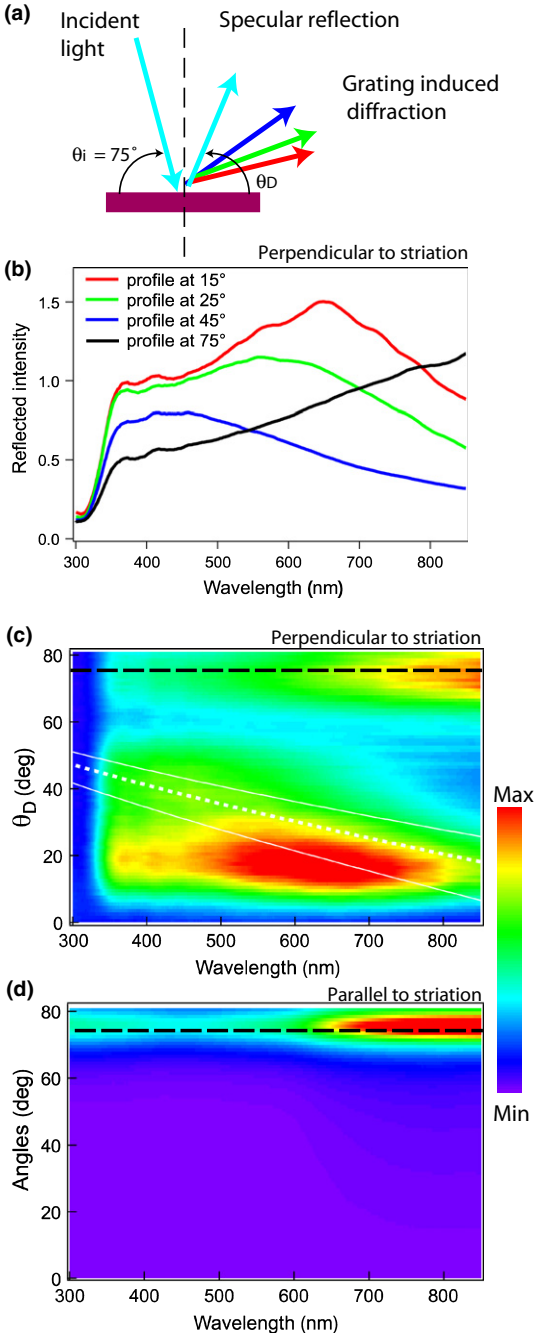

Fig. 2 Optical response of the petal. (a) Schematic view of the experiment. The collimated incident light (spot dimension 1 mm^2) illuminates the petal at an angle of $\theta_i = 75°$. When the direction of the striations is perpendicular to the plane where the detector rotates, part of the light is reflected in the specular direction and other light is diffracted in different directions following the grating formula. (b) Spectra obtained for different angles of collection θ_D showing the iridescent behaviour of the flower (different colours are reflected in different directions). (c) Angular resolved measurements in the same configuration as (a), with striations perpendicular to the detection plane. The intensity of the light scattered from the sample is reported in a rainbow colour-scale as a function of wavelength and angle of detection θ_D. The white lines indicate the position of the diffracted peak for a perfect diffraction grating with spacing 1, 1.3 and 1.6 μm. (d) In a scattering measurement obtained with striations parallel to the detection plane, no scattered signal is observed, but only the specular reflection. All spectra normalized against a white diffuser standard.

In order to prove that this was not an artefact, we measured in the same position the scattering response in configuration (2), that is, with striations parallel to the detector plane. As expected, the diffraction pattern is not present and we can only observe the contribution of the specular reflection (Fig. 2d) again at $\theta_D = 75°$ (black dotted line).

Discussion

The iridescent effect is the result of diffraction from a folded petal cuticle

The cryo-SEM and TEM analyses clearly indicate the presence of striations on the epidermis in the purple region of the petal. (Fig. 1d,e). The average distance between striations is 1.3 ± 0.3 μm (Fig. 1e). The white dotted line in Fig. 2(c) shows the expected position of the first diffraction order calculated using the classical grating formula (Vignolini *et al.*, 2013) and the calculated periodicity of 1.3 μm. The white continuous lines report instead the position of the diffraction order calculated for periodicities of 1.6 and 1 μm (Fig. 2b). The measurements shown in Fig. 2 therefore strongly demonstrate wavelength-dependent reflection for different angles of collection, illuminating a relatively large area of 1 mm^2. Since the dimensions of the cells in the mature stage of the petal are *c.* 15×70 μm, we conclude that the diameter of our illumination spot spans > 60 cells. Consequently, the striations are sufficiently ordered to produce iridescence over a significant area and to enable us to measure it with this configuration. Our technique is in principle analogous to the one used by van der Kooi *et al.* (2014). Optical scatterometry (as used in van der Kooi *et al.*, 2014) provides in only one measurement the directionality of the reflected light in all directions (i.e. an image of the scattered light in the entire hemisphere), while with our optical goniometer we acquire spectrometric data that is, a quantitative measure of the reflected intensity as a function of wavelength for each angle at which we measure, see Vignolini *et al.* (2013).

The iridescent flower is only mature for a short time window

Van der Kooi *et al.* (2014, p. 672) commented that the morphology of the *Hibiscus trionum* flower would prevent any visible iridescent signalling, as the proximal portions of the petals are 'strongly curved inwards towards the centre of the flower . . . reducing the possible illumination angles'. In our growth conditions and others (Buttrose *et al.*, 1977), the petals of *H. trionum* open fully at reproductive maturity (anthesis), allowing illumination from a wide range of possible angles (Fig. 1a,b, note that anthers are dehiscent and have started shedding their pollen). One of the many common names for *H. trionum* is 'flower of an hour', reflecting the rapid development and short lifespan of the mature flower. We have studied the developmental time course of the reproductive organs, the petal, and the petal epidermal structures, and note that the window in which the flower is fully developed is very short (≈ 3 h) (also recorded by Stead & Van Doorn, 1994).

Conclusions

We conclude that iridescence from the flowers of *Hibiscus trionum* is visible with the naked eye under natural light conditions, and that it is possible to capture this effect using an optical goniometer illuminating a large area. The iridescent effect is generated by diffraction from the regularly folded cuticle overlying the petal epidermal cells. The frequency of occurrence of such diffractive gratings across the estimated 300 000 species of angiosperms requires more extensive study, as does the significance of floral iridescent signalling to the foraging behaviour of insect pollinators and thus to the reproductive success of the plant. However, it is unequivocally the case that flowers can and do produce iridescence through diffractive optics.

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