Cuticle chemistry drives the development of diffraction gratings on the surface of *Hibiscus trionum* petals

**Highlights**
- Petal texture is not exclusively set by cuticle production rate and cell expansion
- Color production through ordered striations in *Hibiscus* depends on cuticle chemistry
- Interfering with cuticular chemical composition impairs regular striation formation
- Spatial regulation of cuticle chemistry could restrict buckling to the petal base

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**In brief**
Microscopic striations on petal surfaces can interfere with light and produce structural color. Combining genetic and mechanical manipulations with chemical analysis and theoretical modeling, Moyroud et al. show that to specify where, when, and how striations emerge, *Hibiscus trionum* flowers regulate the chemical composition of their cuticle.
Cuticle chemistry drives the development of diffraction gratings on the surface of Hibiscus trionum petals

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SUMMARY

Plants combine both chemical and structural means to appear colorful. We now have an extensive understanding of the metabolic pathways used by flowering plants to synthesize pigments, but the mechanisms remain obscure whereby cells produce microscopic structures sufficiently regular to interfere with light and create an optical effect. Here, we combine transgenic approaches in a novel model system, Hibiscus trionum, with chemical analyses of the cuticle, both in transgenic lines and in different species of Hibiscus, to investigate the formation of a semi-ordered diffraction grating on the petal surface. We show that regulating both cuticle production and epidermal cell growth is insufficient to determine the type of cuticular pattern produced. Instead, the chemical composition of the cuticle plays a crucial role in restricting the formation of diffraction gratings to the pigmented region of the petal. This suggests that buckling, driven by spatiotemporal regulation of cuticle chemistry, could pattern the petal surface at the nanoscale.

INTRODUCTION

Color is a conspicuous feature of many living organisms, playing important roles in signaling to repel predators and rivals, lure prey, or attract mates. Organisms can produce pigments that absorb subsets of light to generate color or instead produce nanoscale structures made of transparent material to reflect particular wavelengths of light.1–3 These structural colors often produce different hues according to the angle from which the subject is viewed—the phenomenon of iridescence. The minute structures responsible for such colored optical effects are found in both animals and plants. Their morphologies are well characterized and the physical mechanisms underpinning color production in each case have been identified through optical measurements and theoretical simulations.4–6 However, the biological processes controlling the construction of such optical devices during development remain largely unexplored. In plants, structural colors are commonly produced by multi-layer reflectors or by pseudo-regular parallel surface striations acting as diffraction gratings.5,7–9 Diffraction gratings consist of an ordered system of ridges and grooves with a specific periodicity that diffracts light and allows constructive interference for different reflected wavelengths at different angles. How plants develop such structures on their surface remains to be understood.

Here, we use diffraction gratings on petals as an experimental system to address this question. Examples of diffraction gratings have been found in flowers across all angiosperm lineages except the earliest-divergent lineages, and this optical element is likely to have evolved independently multiple times in the flowering plants.10,11 Petal diffraction gratings are made of semi-ordered striations of the cuticle, a waxy polymer that covers the surface of all plant aerial organs.11,12 The presence of disorder in the grating creates weak iridescence and a blue halo—scattered light in the blue-UV part of the spectrum—that is perceived by bumblebees and can enhance their foraging efficiency in laboratory conditions.11

As conventional models do not produce structurally colored flowers, we developed Hibiscus trionum (Malvaceae) as an experimental system to explore diffraction grating development. This species has a short life cycle (seed to flowering in ~7 weeks), it is easy to grow in a range of conditions, readily self-fertilizes, and can also be cross-pollinated. The adaxial petal surface is demarcated into two regions, a white distal region with...
smooth conical and isodiametric epidermal cells and a heavily pigmented purple proximal region where the epidermal cells are flat, elongated (tabular), and topped with parallel cuticular ridges that are partially disordered and create the blue sheen visible at the petal base.10–12

Previous studies have postulated that the striations on the petal surface of *H. trionum* and other species form through mechanical buckling.13–15 Several related but distinct models speculate that the striations develop due to the presence of compressive stresses in the cuticle during petal development. The stress is hypothesized to be generated by conflicting pressures arising from anisotropic growth of the epidermal cells and their walls and simultaneous isotropic production of cuticle by those same cells. The mechanical stress on the cuticle would generate the instability that causes the cuticle to buckle, producing a system of semi-ordered ridges.13–15 Recent work in support of a mechanical model for diffraction grating development showed that applying mechanical stress to isolated *H. trionum* petal tissue can induce the formation of striations in the cuticle of the pigmented region.15

Here, we test the simple model proposed by Antoniou-Kourounioti and colleagues13 and examine whether cuticle production and anisotropic cell growth are sufficient parameters to explain the cuticular patterns found on petal surfaces. We take advantage of the natural diversity of petal surfaces found in different species of *Hibiscus*; we also developed a protocol to transform *H. trionum* and manipulate gene expression *in planta*. Our results indicate that regulating cell growth and the rate of cuticle production is not sufficient to control cuticular patterning. Instead, our results implicate the chemical composition of the cuticle as an essential factor for diffraction grating formation. We propose that the *Hibiscus* petal cuticle could function as a self-organizing material, with chemistry-driven buckling patterning the petal surface at the nanoscale.

**RESULTS AND DISCUSSION**

**Striation formation coincides with anisotropic cell growth**

To start investigating striation formation, we characterized in detail the cell differentiation process on the adaxial epidermis of *Hibiscus trionum* petals throughout bud development (Figure 1A). At stage S1, petal primordia are green, and the epidermal cells appear isotropic and undifferentiated across the entire petal surface (Figures 1B–1D); their cuticle is smooth and anthocyanin pigments are absent, only starting to appear at the very base of the petal in late S1 (Figure 1B). Two distinct

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**Figure 1.** Diffraction gratings emerge halfway through bud development and are restricted to the petal base surface in *H. trionum*

Stereotypical stages of *H. trionum* bud development (S1–S5). S5 is the mature open flower. At S4, petal tips become visible. At S3, the crown of bracts opens and bends toward the horizontal. At S2, pigmentation appears on the petal adaxial epidermis, dividing the petal surface between an anthocyanin-rich proximal region and a non-pigmented distal region. S1 petals are entirely green.

(A) Representative images of a bud at each stage. Scale bars, 0.5 cm.
(B–D) Representative images of a dissected petal at each stage, adaxial epidermis visible (B). Scale bars, 1 mm (S1–S4) and 1 cm (S5). SEM images showing cells in the distal (C) or proximal (D) region of the adaxial petal epidermis at each stage. Scale bars, 20 μm. Differences in cell shape and cuticular patterning appear between S3 and S4. Red arrows indicate the base-to-tip axis of the petal.
regions emerge on the petal surface at S2, as only cells in the proximal region of the petal epidermis accumulate anthocyanin. Cuticular striations are initiated between S3 and S4 and are also restricted to the proximal pigmented region. The unpigmented cells in the distal portion of the petal remain smooth (Figures 1C and 1D). Striation formation is concurrent with anisotropic cell expansion, as epidermal cells in the proximal region start to elongate along the base-to-tip petal axis from S3 onward (Figure 1D). Cells in the distal region instead mostly expand perpendicularly to the petal surface, acquiring their characteristic conical shape. Thus, cuticular striations emerge simultaneously with anisotropic cell growth.

Measurement of cuticle thickness and cell expansion is insufficient to predict cuticular pattern

The early model proposed by Antoniou-Kourounioti and colleagues describes how different degrees of anisotropic cell elongation and cuticle production result in mechanical stress. Specifically, the model proposes that where the amount of cuticle produced keeps pace with cell growth and cuticle thickness remains constant, the cuticle layer will be stress-free, but when the cuticle production rate is inconsistent with cell growth, then compressive or tensile stresses will occur. To release this stress, the cuticle buckles and forms striations. To test whether a change in cell expansion (in magnitude and direction) would lead to a change in cuticular pattern, as predicted by the model, we first applied chemical treatments to developing Hibiscus trionum buds before striation formation (<S3) and recorded the cuticular patterns observed once flowers open (S5). Ectopic application of cytoskeletal drugs or plant hormones successfully modified cell expansion in the proximal region of the petal epidermis, but striations were always produced, regardless of changes in cell dimension (Figure S1).

This result is consistent with the predictions of the theoretical model (Figure 2A) and confirms experimentally that cuticular pattern topology is resistant to cell growth variations when those
fall within the range defined by the model. As chemical treatments did not allow us to cross model boundaries, we used natural diversity to enhance our exploration of the possibility space and access other cell behaviors (Figures 2B and 2C). We selected different accessions and species from the Trionum and Phylloglandula sections of *Hibiscus* (Data S1) and compared the cuticular pattern observed in the proximal region of the adaxial petal epidermis with the pattern predicted by the model. According to the model, the parameter values measured in all cases (cell expansion and cuticle production; Table S1) should induce striation formation (Figures 2D–2F). However, the petal epidermal cells of *H. sabdariffa*, *H. cannabinus*, and *H. trionum* (Botswana accession) are all completely smooth (Figure 2B). Therefore, we conclude that the cell growth and change in cuticle thickness recorded in related *Hibiscus* species does not always generate the predicted pattern (Figure 2D and 2E). This result indicates that the model of Antoniou-Kourounioti and colleagues is not sufficient to predict petal cuticle patterning in *Hibiscus*.

**Interfering with cuticle production is sufficient to modify cuticular patterning**

To determine whether varying cuticle production could affect diffraction grating assembly, we used a transgenic approach to misexpress genes encoding enzymes or transcriptional regulators contributing to the synthesis or assembly of cuticular components in other species. First, we interfered with cuticle structure by constitutively expressing CUTCILE DESTRUCTING FACTOR1 from Arabidopsis, AtCDEF1 (Figure S2A). AtCDEF1 encodes a GDSL lipase-esterase that disrupts cuticle integrity when overexpressed in Arabidopsis.\(^{16}\) Flowers from *Hibiscus trionum* 35S:AtCDEF1 appear similar to wild type in terms of petal size/shape, pigmentation pattern, and cell shape, but lack the bluish tinge in the proximal region of the petal (Figures 3A, 3D, and 3G). Closer examination revealed that the striated portion of the petal was greatly reduced and discontinuous, with cuticular ridges found only on irregular patches of cells toward the petal base (Figures 3B, 3C, 3E, and 3F versus Figures 3H and 3I). Thus, impairing ordinary cuticle assembly is sufficient to disrupt diffraction grating formation in *H. trionum*. Reciprocally, we also aimed to evaluate the effects of cuticle overproduction on epidermal texture. In Arabidopsis thaliana, the SHINE1 transcription factor from the AP2/EREBP-like superfamily plays a key role in driving cuticle production.\(^{17–19}\)

The SHINE A lineage to which Arabidopsis SHINE1 belongs appears to be lost in Malvaceae, as we found no orthologs in the genomes of cotton (*Gossypium raimondii*) or cocoa (*Theobroma cacao*) (Figure 3J; Data S2). However, we identified three SHINE-like genes in *H. trionum*. Our phylogenetic analysis (Figure 3J) shows that *HtSHINE1* belongs to the SHINE B clade, which also contains the other two Arabidopsis homologs, *SHINE2* and *SHINE3*. The remaining two *Hibiscus SHINE* genes belong to the SHINE C lineage, which has no Arabidopsis representative (Figure 3J). To identify which *Hibiscus SHINE*, if any, was most likely to control cuticle production where the diffraction grating forms, we used qRT-PCR to follow their expression throughout petal development (Figure 3K). We found that *HtSHINE1* (**HtSHN1**) and *HtSHINE2* (**HtSHN2**) are both expressed at very low levels across the petal, and by S3, corresponding transcripts are barely detectable in the proximal region, where striations are about to initiate. In contrast, *HtSHINE3* (**HtSHN3**) transcription is restricted to the proximal region and increases dramatically from S2 to S4, reaching expression levels two hundred times higher than the maximum levels recorded during petal development for the other two *HtSHINE* homologs (Figure 3K). The spatiotemporal expression pattern of *HtSHN3* makes it a strong candidate for regulating cuticle production at the petal base as diffraction gratings emerge. To test whether HtSHN3 was able to induce striation formation, we expressed *HtSHN3* constitutively in *H. trionum* using the Arabidopsis *UBQ10* promoter (Figure S2B). During the vegetative phase, UBQ10:*HtSHN3* plants showed characteristic signs of cuticle defects, including slower growth and smaller leaves with curled margins (Figure S3A), also observed in Arabidopsis when *SHINE1* is overexpressed.\(^{16}\) Once plants started to flower, constitutive expression of *HtSHN3* was sufficient to trigger striation formation in the cuticle of some epidermal cells that are ordinarily smooth in wild type. On the adaxial petal epidermis of UBQ10:*HtSHN3* flowers, the tabular smooth cells located in the transition zone between the purple and the white part of the petal occasionally developed a striated cuticle (Figure 3L, compared with Figure S3B). Ectopic striations were also recorded on the usually smooth abaxial petal surface (Figures S3C and S3D), covering individual patches of tabular and puzzle cells in the proximal and distal regions, respectively (Figures 3M and 3N). Taken together, these results indicate that constitutive expression of *HtSHN3* can induce ridge emergence on otherwise smooth cells, but the effects are uneven: only small groups of isolated cells gain striations while the surface of most cells remains unchanged (Figures 3L–3N).

To modify cell surface patterning more extensively across the petal, we fused the strong viral activation domain VP16\(^2\) to the transcriptional silencing motif SRDX (EAR repressor motif of Arabidopsis SUPERMAN\(^1\)) to *HtSHN3*. Surprisingly, when overexpressed in *Hibiscus* (Figures S2E and S2F) both fusion proteins generated identical phenotypes, characterized by complete lack of striation in the proximal region of the petal (Figures 4A–4F). This is not due to compensatory ectopic expression of *HtSHN1* or *HtSHN2* in the proximal region of the petal, as qRT-PCR confirmed that both genes remain expressed at very low levels (Figures S2H and S2I). To gain insight into the underpinning mechanisms accounting for these phenotypes, we examined the expression of potential target genes of *HtSHN3* in both types of transgenic lines. Transcriptomic analyses in Arabidopsis identified genes whose expression is affected when *SHN1* or *SHN1-SRDX* is overexpressed.\(^{22}\) These genes include members of the cytochrome P450 family 77 (i.e., *At3g10570*) and genes coding for GDSL-motif lipases/hydrolases (i.e., *At2g04570*). We isolated *Hibiscus trionum* sequences homologous to *At3g10570* (**HtCYP77**) and *At2g04570* (**HtGDSL**) and compared their expression levels in wild type and lines overexpressing *HtSHN3-SRDX* or *HtSHN3-VP16* (Figure 4G). Both *HtCYP77* and *HtGDSL* are downregulated in 3SS:*HtSHN3-SRDX*, consistent with results obtained in Arabidopsis. Contrary to our initial assumption, *HtCYP77* and *HtGDSL* are also downregulated in plants overexpressing *HtSHN3-VP16* (Figure 4G). This could explain why both types of transgenic lines exhibit the same lack-of-striation phenotype. We hypothesize that the addition of the VP16 domain may have impaired the ability of *HtSHN3* to activate transcription by...
influencing its folding or its ability to interact with DNA or other proteins. Taken together, our data indicate that interfering with cuticle production in *Hibiscus* is sufficient to modify surface texture in the petal epidermis.

The chemistry of the petal cuticle is a key factor controlling pattern formation

The cuticular pattern variations we observed after manipulating *HiSHN3* expression and/or activity could be due to quantitative (cuticle thickness) or qualitative (cuticle components) changes. Indeed, previous studies in Arabidopsis and Tomato have shown that both the amount and composition (components of and relative amounts of cutin, waxes, and other secondary metabolites) of the cuticular layer are altered when *SHN-like* genes’ expression levels are modified.18,23

To examine whether striation absence coincides with a reduction in cuticle synthesis during *Hibiscus* petal development, we used a Fat Red assay to stain the cuticle of petal cross-sections...
Figure 4. Cuticular striations can be lost without changing cell shape or cuticle thickness

(A–D) Floral phenotype of 5S:HtSHN3-VP16 H. trionum transgenic lines. (A) Representative flower showing the absence of blue sheen in the flower center. (B) Close-up view of the proximal region of the adaxial petal epidermis. (C and D) Light (C) and SEM imaging (D) of cells from the epidermis region shown in (B). Striations are absent and cells are smooth.

(E and F) Floral phenotype of 35S:HtSHN3-SRDX H. trionum transgenic lines. (E) Light imaging and (F) SEM imaging of the cells from the proximal region of the adaxial petal epidermis. Striations are absent and cells are smooth.

(G) Relative expression levels of HtGDSL and HtCYP77 in the proximal region of developing petals of H. trionum wild type, 35S:HtSHN3-SRDX, and 35S:HtSHN3-VP16 at S3 and S4. *p < 0.05, **p < 0.02, and ***p < 0.001, Welch’s t test. Error bars represent SD.

(H and I) Light imaging of cross-sections of petal epidermis (adaxial proximal region) from H. trionum wild type (H) and 35S:HtSHN3-VP16 transgenic line stained with FatRed (I). The cuticle appears pink.

(J and K) Model prediction of cuticular pattern as in Figure 1 with (J) β = 0.85 (solid lines) for H. trionum wild type (black asterisk), β = 0.685 (dashed lines) for 35S:HtSHN3-VP16 (hollow red circle), and (K) β = 1.05 for 35S:HtSHN3-SRDX (solid red circle) and 35S:HtMIXTA-like1 (hollow red star). Red indicates discrepancy between observed and predicted patterns.

(L–P) H. trionum transgenic lines constitutively expressing HtMIXTA-like1 (35S:HtMIXTA-like1).

(L) Representative flower (line #1) showing the lack of blue sheen in the flower center.

(M and N) Close-up view of the proximal region of the adaxial petal epidermis from line #1 and #2, respectively. A blue patch (dotted red line) coincides with residual striations. The images represent the two extremes in terms of size of the striated patch.

(O and P) Light (O) and SEM imaging (P) of epidermis cells from the region shown in (M). Most cells have a smooth cuticle, but residual faint striations are visible on some cells on the left.

Scale bar, 2 mm (B, M, and N), 100 μm (C), 25 μm (D and F), 40 μm (E and P), 5 μm (H and I), and 50 μm (O).

See also Figures S1, S2, S4, and S6 and Table S1.
in the proximal region of wild-type and 3SS:HtSHN3-VP16 lines. This revealed no significant difference in cuticle thickness at stage 5 (Figures 4H and 4I), suggesting that other regulators of cuticle synthesis have a greater influence on cuticle production rate. Similarly, CryoSEM fractures of the petal adaxial epidermis proximal region did not uncover any difference in architecture of the cuticle and cell wall between wild-type and HtSHN3-VP16 or HtSHN3-SRDX lines (Figure S4). Next, we estimated cuticle production rate (STAR Methods; Figures S1H–S1J), which measures whether cuticle growth keeps pace with cell growth. There were differences in cuticle thickness at S3, which resulted in some variation in the cuticle production rate between lines and, therefore, the predicted stresses. We also estimated the expansion experienced by cells in the proximal region of the petal adaxial epidermis in the various HtSHN3 transgenic lines (Figures 4J and 4K) and used our theoretical model to infer the expected surface pattern. According to the model, proximal tabular cells from these transgenic lines should all develop cuticular striations, despite small changes in the β and expansion parameters (Figures 4J and 4K), but they do not. Such discrepancies between predicted and observed patterns are reminiscent of our earlier observations for other Hibiscus species (Figures 2B, 2D, and 2E). Developmental models try to simplify processes occurring in living tissues. When they are proven incorrect by experimental data, this often indicates that parameters are missing and/or that the model is too simple. For instance, the theoretical model we used does not take into account the nature of the cuticular material. As SHINE homologs could also influence cuticle composition, we hypothesized that the absence of striations, even when the conditions should induce buckling according to the model, could be due to a change in cuticle chemical components. This would modify the mechanical properties or structure of the cuticle, altering the system behavior under compressive stresses and its propensity to buckle.

To test this hypothesis, we used a transgenic approach to alter cuticle chemistry. MYB106 and MYB16, members of the MIXTA-like class of MYB transcription factors, act redundantly in Arabidopsis to induce wax biosynthesis. Waxes are important components of the cuticle and are likely to reduce water permeability on the plant surface. We isolated a MIXTA-like gene, HtMIXTA-like1, from H. trionum petals and used the strong CaMV 35S promoter to drive its expression constitutively in planta (Figure S2C). The metallic blue sheen characteristic of the petal proximal region appeared greatly reduced in 35S:HtMIXTA-like1 (35S:HtML1) flowers (Figure 4L). This also coincides with the disappearance of striations: the cuticle overlaying the pigmented epidermal cells in 35S:HtML1 plants appears mostly smooth with only patches of striated cells remaining (Figures 4M–4P). When present, striations are shallow and less prominent than in wild type (Figures 4O and 4P).

Studies in Arabidopsis have also identified a second regulator of wax production, Decrease Wax Biosynthesis (DEWAX), an AP2/ERF factor that transcriptionally represses wax biosynthetic genes. To alter wax production, we introduced a 35S:AtDEWAX construct into Hibiscus trionum (Figure S2G). The resulting plant showed no obvious phenotypic defect except for disruption of diffraction grating formation (Figures 5A–5C). In wild type, cuticular
striations are continuous between epidermal cells so that individual cells are hard to distinguish (Figure S5D; see also Figure 1D stage 5 or Figure 3I). However, in the 3SS:ADEWAX line, cell outlines are extremely prominent, with the cuticle accumulating over the cell-cell junctions (Figures 5A and 5B). Striations within a cell area were sometimes completely missing, but most often misaligned (Figure 5B) or severely disrupted (Figure 5C). The observation that altering wax content disrupts striation development has not been made in Arabidopsis, possibly as a result of the different chemistries and material properties of different cuticles.

Cutin is a polyester of hydroxy fatty acids that forms a matrix in which compounds, including waxes and phenolics, are embedded.\(^{33}\) The \textit{CUTIN DEFICIENT1} (CD1) gene from Tomato and its homologs in Arabidopsis belong to a family conserved across land plants coding for cutin synthase-like (CUS) proteins that catalyze the formation of cutin oligomers.\(^{27-29}\) In Arabidopsis, CUS2 is necessary for the maintenance of the irregular cuticular ridges that usually cover sepal.\(^{30}\) We isolated a CUS family member from our \textit{H. trionum} petal cDNA library, \textit{HTCUS1}, and produced a series of transgenic lines overexpressing \textit{HTCUS1} (Figure S2D). These plants showed phenotypes typically associated with cuticular defects, such as fusion between neighboring organs (Figure S5A). They also showed a loss of conspicuous blue sheen at the flower center (Figures SSD and SSE). Close inspection of the petal surface confirmed that diffraction grating formation is impaired: when present, cuticular striations are faint and interrupted, and often short and restricted to the junction between neighboring cells (Figures 5E and 5F compared with Figures 5G and 5F–5N). This reduction in cuticular striation is not petal-specific, as the disordered striations that cover the adaxial side of wild-type \textit{H. trionum} leaves (Figure S5B) are mostly absent in 3SS:\textit{HTCUS1} plants (Figure S5C).

Our results point toward a key role for cuticle composition and/or the arrangement of its constituents in diffraction grating formation. CUS gene silencing in tomato led to a decrease in cutin density caused by a reduction in ester bond formation and the presence of nanopores in the polymer.\(^{33}\) Therefore, CUS overexpression may change the degree of cutin reticulation: additional cutin synthase activity could lead to the production of a more densely cross-linked polymer and, thus, a cuticle with distinct mechanical properties, in particular a different degree of stiffness.\(^{38,39}\) Our conclusion that the presence of dihydroxy-palmitic acid may be a powerful predictor of the presence/absence of striation.\(^{40}\) Other compounds, such as C\(_{32}\)H\(_{56}\)O\(_{4}\), also appear to make significant contributions to PC1 (Table S2A). However, although C\(_{32}\)H\(_{56}\)O\(_{4}\) is almost always detected in samples with a striated cuticle, it is also present in almost half of the samples with a smooth surface (Table S2A). Thus, C\(_{32}\)H\(_{56}\)O\(_{4}\) seems a less powerful predictor of the presence/absence of striation.

Next, we set out to characterize the chemical composition of a second independent set of cuticles by LESA-MS analyses on the petal surfaces of the different species of \textit{Hibiscus} examined in Figures 2B and 2C. We expanded this sample set by characterizing two \textit{H. trionum} samples without a diffraction grating: (1) the abaxial (underneath) petal epidermis from mature \textit{H. trionum} flowers and (2) the adaxial surface of stage-3 petals that are yet to form cuticular striations (Figure 1D, stage 3). Here too, PCA identified a first PC that separates striated from smooth cuticular samples (Figure 6B). Similar to the observed result when using transgenic lines as samples (Table S2A), wax accumulation appears to characterize the striated cuticles while potentially phenolic compounds associate with smooth surfaces (Table S2B; Figures 6C and 6D). We also noted that half of the top 20 compounds are shared by both datasets (Table S2), when the compounds detected were ranked in each set according to their respective contribution to PC1 (Figures 6A and 6B). Probable phenolic compounds C\(_{27}\)H\(_{52}\)O\(_{19}\) and C\(_{27}\)H\(_{52}\)O\(_{18}\), the cutin monomer dihydroxy-palmitic acid and six potential waxes all show association with one type of cuticular pattern (smooth or striated), thus, modifying cuticle chemistry, and possibly the chemistry of the entire cell-wall-cuticle continuum, could be one of the mechanisms at work to modify cuticle patterning.
Changes targeting the biosynthetic pathways that produce these compounds could arise during evolution, triggering loss or gain of diffraction gratings in different Hibiscus lineages and accounting for the lability of iridescence across the genus. Regulatory changes affecting the same pathways could also occur during development from stage S3 onward, to produce a cuticular material whose mechanical properties are suitable for diffraction grating formation on the surface of a growing petal. Previous work showed that striation formation via buckling can be induced by applying a mechanical force to the smooth proximal region (Figure 1D) of S3 H. trionum petal primordia (pre-striation stage). To test whether differences in chemical constituents between striated and smooth cuticles correlate with differences in mechanical properties, we used a similar procedure to that described in the study conducted by Airoldi et al. to apply in-plane mechanical stress to the proximal region of S3 pre-striation stage petals from transgenic Hibiscus lines with a smooth surface (35S:HtSHN3-VP16 and 35S:HtSHN3-SRDX) and wild type for comparison (Figure S6). Our results show that, while prominent cuticular ridges readily appeared on the surface of all pre-striation stages in wild-type samples upon application of mechanical perturbation (Figure S6A), cuticular striations failed to form on the surface of both 35S:HtSHN3-VP16 and 35S:HtSHN3-SRDX S3 petal primordia when an equivalent force was applied (Figures S6B and S6C). In wild-type plants, striations are clearly visible on stage S4 petal primordia (Figure 1D) but absent from 35S:HtSHN3-SRDX and 35S:HtSHN3-VP16, as the transgenic lines produce petals with an entirely smooth adaxial surface (Figures 4A–4F and S6D). The application of an external force to S4 petal primordia from plants overexpressing HtSHN3-VP16 was not sufficient to restore a wild-type phenotype, as most stretched cells remained smooth, with some cells occasionally exhibiting very short and faint striations (Figure S6D). Taken together, our results indicate that equivalent petal tissue regions, with similar cell geometry, dimensions, cuticle thickness, and architecture (Figures 4C–4F, 4H, 4I, and S4) but different cuticle chemical profiles, can vary in their buckling ability and thus their capacity to form a diffraction grating during development or upon application of a mechanical force.

Figure 6. Striated cuticles share a common chemical profile

(A and B) Principal component analyses of cuticle chemical composition characterized by LESA-MS of wild-type and transgenic lines of (A) H. trionum or (B) H. trionum and related species from Figures 2B and 2C. Each sample type is represented by a symbol with a number. The number indicates the species/line and petal region analyzed and the symbol indicates whether the sample surface is striated or smooth. Samples 1 and 2 are identical, included in both analyses for reference. In each plot, regions occupied by smooth and striated samples are shaded with yellow and purple, respectively.

(C and D) Comparison between the chemical composition (shortlisted compounds only, plotted according to double bond equivalent [DBE] versus the number of carbons |C| of (C) the smooth surface of petal primordia (S3, proximal and distal regions) and the striated surface of open flower petals (S5, proximal region) or (D) striated and smooth petal surfaces in open flowers from different species or populations. Symbols corresponding to compounds detected on the surface of both types of striated cuticles, and only there, have been colored pink. The regions of the plot occupied by smooth and striated samples are shaded in yellow and purple, respectively (overlap appears brown).

See also Figure S6, Table S2, and Data S1 and S4.
LESAs-MS data from this study (Figure 6) and from Giorio et al. demonstrate distinct chemical profiles between the striated and non-striated portions of the same petal; we have also shown the spatially restricted expression of a key transcription factor, HsHINES, involved in the production of cuticle components in the proximal region of the petal (Figure 3K). Previous studies have reported distinct cuticular properties of different epidermal cell types, but our work indicates that cuticle chemistry can be controlled with exquisite spatial resolution across fields of cells within a single organ, allowing different regions of the same epidermis to exhibit different mechanical behaviors and surface textures that themselves cross boundaries of multiple cells.

**Conclusions**

Here, we demonstrate experimentally that cell surface patterning in petals is resistant to cell growth variations that fall within the range predicted by theoretical models. However, the cuticle texture emerging on the surface of developing petal primordia cannot be predicted by our early theoretical model, which proposed that the final buckling outcome is simply a product of the rate of cuticle production and the extent and direction of epidermal cell expansion.

Our study reveals that the formation of ordered striations, and thus the production of structural colors, is strongly dependent on the chemical composition of the cuticle itself. We show that cuticular wrinkling in multiple Hibiscus species is associated with the presence of a high level of dihydroxy-palmitic acid and waxes and the exclusion (or reduced levels) of probable phenolic compounds. We have also demonstrated, using a transgenic approach in a new model system, that interfering with the production of these compounds can impair striation formation in Hibiscus trionum to various degrees, both during development or upon application of a mechanical force. Chemical changes such as these may alter the behavior of the cuticle under compressive forces through changes in its material properties. For example, the viscosity of the cuticle (which may result from its degree of polymerization and its relative wax and lipid content), its strength and its stiffness (or Young’s modulus) may all be impacted. Previous studies have demonstrated the particular importance of relative stiffness in the wrinkling behavior of layered systems. The chemical composition of the cuticle could also affect its organization, producing architectures that are more or less favorable to buckling. The relationship between cuticle chemical composition and its ultrastructure is not fully understood, but the nature of the molecules delivered to the cell surface, as well as their timing and rate of delivery, are all likely to influence the layered organization of the cuticular membrane.

More recent models than the one tested here propose that the cuticle of Hibiscus behaves as a bilayer, with a relatively stiff thin film on a soft substrate. The data reported here are compatible with this model; without testing it explicitly, the results of the mechanical perturbations suggest that a change in cuticle chemistry could be sufficient to change its buckling propensity. It will be interesting in future studies to investigate whether a change in cuticle chemistry is sufficient to trigger a change in the formation of a bilayered structure, or of its mechanical properties, or both. Such studies will be necessary to establish the exact contribution of chemistry to the resulting material properties of the cuticle, but our results show that flowering plants can control precisely the position and size of diffraction gratings on their petals by specifying where distinct biosynthetic pathways are active across the epidermis. Floral diffraction gratings are pseudo-ordered, and the presence of disorder plays a key role in shifting the optical effect toward the blue/UV end of the spectrum visible to insect pollinators. It remains to be established whether cuticle chemistry can also fine-tune cuticular mechanical properties with sufficient accuracy to control the regularity of the grating itself.

**STAR METHODS**

Detailed methods are provided in the online version of this paper and include the following:

- **KEY RESOURCES TABLE**
- **RESOURCE AVAILABILITY**
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**SUPPLEMENTAL INFORMATION**

Supplemental information can be found online at [https://doi.org/10.1016/j.cub.2022.10.065](https://doi.org/10.1016/j.cub.2022.10.065).

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**AUTHOR CONTRIBUTIONS**

E.M. and B.J.G. designed the study. E.M., C.A.A., J.F., C.G., S.S.S., M.K., P.J.R., C.J.P., S.H., J.F.W., and S.R. performed the experiments and analyzed the data as follows: E.M. isolated the different *H. trionum* genes, built the different plant expression vectors, and developed the transformation protocol.
for \textit{H. trionum}: E.M. and C.A.A. produced, imaged, and analyzed the transgenic Hibiscus lines; E.M., S.H., C.A.A., and J.F. measured cell expansion and cuticle thickness; C.A.A. performed and analyzed the qPCR experiments; C.J.P. and P.J.R. performed and imaged the FatRed staining of the cuticle; C.G. and S.S.S. performed the LESA-MS analyses and analyzed the data with M.K.; J.F.W. performed the phylogenetic analyses; J.F. performed the PCA analyses; S.R. and E.M. performed the mechanical perturbation experiments; and E.M. prepared the figures and wrote the manuscript with B.J.G. All authors read and commented on successive drafts of the manuscript and approved the final version.

DECLARATION OF INTERESTS

B.J.G. is a member of the advisory board of \textit{Current Biology}.

INCLUSION AND DIVERSITY

We support inclusive, diverse, and equitable conduct of research.

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## STAR METHODS

### KEY RESOURCES TABLE

| REAGENT or RESOURCE                          | SOURCE                      | IDENTIFIER  |
|----------------------------------------------|-----------------------------|-------------|
| **Bacterial and virus strains**              |                             |             |
| Escherichia coli DH5α competent cells        | ThermoFisher                | Cat# 18265017 |
| ElectoMAX Agrobacterium tumefaciens LBA4404  | ThermoFisher                | Cat# 18313015 |
| **Chemicals, peptides, and recombinant proteins** |                             |             |
| Phusion High-Fidelity DNA Polymerase         | New England Biolabs         | M0530L      |
| EcoRV                                        | New England Biolabs         | R0195S      |
| EcoRI                                        | New England Biolabs         | R0101S      |
| T5 Exonuclease (for Gibson Assembly)        | New England Biolabs         | M0663S      |
| Taq DNA ligase (for Gibson Assembly)        | New England Biolabs         | M0208S      |
| Plant Agar                                   | Sigma-Aldrich               | A7921       |
| Kanamycin                                    | Sigma-Aldrich               | BP861       |
| Streptomycin                                 | Sigma-Aldrich               | S9137       |
| MS salts with vitamins                       | Duchefa                     | M0222       |
| Acetosyringone                               | Sigma-Aldrich               | D134406     |
| L-cysteine                                   | Sigma-Aldrich               | 168149      |
| L-Ascorbic acid                              | Sigma-Aldrich               | A4544       |
| Thidiazuron (TDZ)                            | Sigma-Aldrich               | P6186       |
| 6-Benzylaminopurine (BA)                    | Sigma-Aldrich               | B3408       |
| Cefotaxime                                   | Sigma-Aldrich               | C7039       |
| Glutaraldehyde                               | Sigma-Aldrich               | G7651       |
| Sudan Red 7B                                 | Sigma-Aldrich               | 201618      |
| Lanolin                                      | Sigma-Aldrich               | L7387       |
| 3-indoleacetic acid (IAA)                    | Sigma-Aldrich               | I3750       |
| Gibberelic acid, 98%                         | Fischer scientific          | 15402377    |
| NPA                                          | Sigma-Aldrich               | PS343       |
| Taxol                                        | Sigma-Aldrich               | PHL89806    |
| Oryzalin                                     | Sigma-Aldrich               | 36182       |
| Formic acid                                  | Sigma-Aldrich               | 695076      |
| Acetonitrile                                 | Sigma-Aldrich               | 34851       |
| Chloroform                                   | Sigma-Aldrich               | 366927      |
| **Critical commercial assays**               |                             |             |
| Spectrum Plant Total RNA kit                 | Sigma-Aldrich               | STRN50      |
| SuperScript III First-strand synthesis system| Invitrogen                  | 18080051    |
| SuperScript II Reverse Transcriptase         | Invitrogen                  | 18064071    |
| Luna Universal qPCR Master Mix               | New England Biolabs         | M3003L      |
| **Deposited data**                           |                             |             |
| HtSHINE1 sequence                            | GenBank                     | MW801439    |
| HtSHINE2 sequence                            | GenBank                     | MW801440    |
| HtSHINE3 sequence                            | GenBank                     | MW801441    |
| HtMIXTA-like1 sequence                       | GenBank                     | MW801437    |
| HtCUS1 sequence                              | GenBank                     | MW801438    |
| **Experimental models: Organisms/strains**   |                             |             |
| Hibiscus trionum, L.                         | Chiltern seeds (UK)         | Cat No. 688K |
| Hibiscus sabdariffa                          | Chiltern seeds (UK)         | Cat No. 688A |
| Hibiscus panduriformis                       | Sunshine seeds (Germany)    | N/A         |

(Continued on next page)
### REAGENT or RESOURCE SOURCE IDENTIFIER

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Hibiscus cannabinus | Jungle seeds (UK) | JS628 |
| Hibiscus trionum (commercial) | van der Kooi et al. | N/A |
| Hibiscus richardsonii (Mayor Island (Tuhua), New Zealand) | Murray et al. | Voucher AK251841 |
| Hibiscus trionum 'New Zealand 'naturalised race' (Bream Head, North island, New Zealand) | Murray et al. | Voucher AK253689 |
| Hibiscus trionum (Botswana) | Millennium Seed Bank | Acc. Nb 91154 |
| Hibiscus trionum, L: 2xp35S::AtCDEF1 | This study | N/A |
| Hibiscus trionum, L: 2xp35S::AtDEWAX | This study | N/A |
| Hibiscus trionum, L: 2xp35S::HtSHN3-VP16 | This study | N/A |
| Hibiscus trionum, L: 2xp35S::HtSHN3-SRDX | This study | N/A |
| Hibiscus trionum, L: 2xp35S::HtMIXTA-like1 | This study | N/A |
| Hibiscus trionum, L: 2xp35S::HtCUS1 | This study | N/A |
| Hibiscus trionum, L: pAtUBQ10::HtSHN3 | This study | N/A |

### Oligonucleotides

| Oligonucleotides | See Data S3 for Oligonucleotides | N/A | N/A |
|------------------|----------------------------------|-----|-----|

### Recombinant DNA

| Recombinant DNA | Modified pGreen II 0229 with 2xp35S | This study | N/A |
|-----------------|-------------------------------------|------------|-----|
|                  | Modified pGreen II 0229 with 2xp35S and pUBQ10::eYFPmyr | This study | pEM110 |
|                  | AtCDEF1 in pBluescript KS(-) | This study | pEM111 |
|                  | 2xp35S::AtCDEF1 in pEM110 | This study | pEM112 |
|                  | HtSHN3 in pBluescript KS(-) | This study | pEM75 |
|                  | pAtUBQ10::HtSHN3 in pGreen II 0229 | This study | pEM76 |
|                  | 2xp35S::HtSHN3-VP16 in pEM110 | This study | pEM114 |
|                  | 2xp35S::HtSHN3-SRDX in pEM110 | This study | pEM115 |
|                  | HtMIXTA-like1 in pBluescript KS(-) | This study | pEM85 |
|                  | 2xp35S::HtMIXTA-like1 in pEM110 | This study | pEM109 |
|                  | HtCUS1 in pBluescript KS(-) | This study | pEM99 |
|                  | 2xp35S::HtCUS1 in pGreen II 0229 | This study | pEM100 |
|                  | AtDEWAX pBluescript KS(-) | This study | pEM97 |
|                  | 2xp35S::AtDEWAX in pGreen II 0229 | This study | pEM98 |

### Software and algorithms

| Software and algorithms | ImageJ | Abramoff et al. | https://imagej.nih.gov/ij/ |
|-------------------------|--------|-----------------|-----------------------------|
|                         | ACME controller software ACMERobotX | Robinson et al. | https://github.com/ACME-Robinson/InstallPackage |
|                         | BLASTP | Altschul et al. | N/A |
|                         | MAFFFT v7.271 | Katoh et al. | N/A |
|                         | phyx v.0.999 | Brown et al. | N/A |
|                         | iQtree v1.6.3 | Nguyen et al. | N/A |
|                         | R | R Core Team | N/A |
|                         | ade4 package | Dray and Dufour | N/A |
|                         | Xcalibur 2.1 | Thermo Scientific, and Zielinski et al. | N/A |
|                         | Mathematica 10 | Wolfram research, UK and Giorio et al. | N/A |

### Other

| Other | Keyence VHX-5000 microscope with a VH-Z20, a VH-Z100 and a VH-Z500 lenses | Keyence | N/A |
|-------|-------------------------------------------------------------------------|--------|-----|
|       | Automated Confocal Micro-Extensometer (ACME) | Robinson et al. | N/A |
|       | Tough tags | DiversifiedBiotech | TTSW-1000 |

(Continued on next page)
RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources should be directed to and will be fulfilled by the lead contact, Edwige Moyroud (em500@cam.ac.uk).

Materials availability
Plasmids used to generate transgenic plant material are available upon request. Seeds from fertile transgenic lines harbouring T-DNAs from those plasmids are available upon request. This study generated no other new unique reagents.

Data and code availability
- The accession numbers of the sequenced genes reported in this paper are GenBank: MW801437, GenBank: MW801438, GenBank: MW801439, GenBank: MW801440 and GenBank: MW801441. The Phylogenetic tree for AP2/ERF can be found in Data S2. Primers are listed in Data S3. The frequency of detection for each of the 135 compounds used for a target compound search in all Hibiscus samples (wild type and transgenic lines of H. trionum and various other Hibiscus species) following LESA-MS analyses are provided in Data S4.
- This paper does not report original code and all software and algorithms are available from the references listed in the key resources table.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Plant material and growth conditions
Plants were grown under glasshouse conditions on a 16 hours of light, 8h of dark photoperiod at 23 °C, minimum radiance 405 μmol.m⁻².s⁻¹, in Levington’s M3 (UK) compost using H. trionum L. seeds (H. trionum, wild type) and H. sabdariffa seeds from Chiltern seeds (UK); H. panduriformis form Sunshine seeds (Germany) or H. cannabinus seeds purchased from Jungle seeds (UK). H. trionum commercial seeds from van der Kooi et al.45 were supplied Doekele G. Stavenga. H. richardsonii (Mayor Island (Tuhua), New Zealand - Voucher AK251841) and H. trionum New Zealand ‘naturalised race’ (Bream Head, North Island, New Zealand - Voucher AK253689) were supplied by Brian G. Murray.46 Seeds from H. trionum, Botswana were obtained from the Millennium Seed Bank (Accession number 91154). Plants received supplemental lighting from Osram 400 W high-pressure sodium lamps (Osram, München, Germany) during the growth period.

METHOD DETAILS

Plant tissue collection
Petal tissues were harvested from developing buds (stage 1-5), between 9:00 am and 11:00 am when stage 5 flowers are fully open. Petals from stage 2 to 5 were dissected to separate the proximal region (purple) from the distal region (white) and immediately frozen in liquid nitrogen. Leaves and Stage 1 petals were frozen in liquid nitrogen without any dissection. All tissue samples were stored at -80 °C until RNA extraction.

Gene isolation
The full-length coding sequences of HtSHINE1, HtSHINE2, HtSHINE3, HtMIXTA-like1 and HtCUS1 were isolated from H. trionum mixed buds cDNA, using a combination of PCR with degenerate primers, 3’ RACE and CELI-PCR.56 Degenerate primers (Data S3) were designed to match regions conserved between homologs from cotton (Gossypium raimondii) and cocoa (Theobroma cacao), two Malvaceae species whose sequenced genomes can be freely accessed via Phytozome v11.0. The full-length coding sequences of AtDEWAX (At5G61590) and AtCDEF1 (At4G30140) were amplified by PCR from A. thaliana (Col-0) bud and leaf cDNA.
Construction of plant expression vectors

The coding sequence of \textit{AtCDEF1} was amplified by PCR using Phusion High-Fidelity DNA Polymerase and primers oEM234F x oEM235R (Data S3). The PCR product was cloned in the pBlueScript KS(-) vector backbone using the EcoRV site to generate the intermediate vector pEM111. Traditional cloning following restriction digest by EcoRI was used to extract the coding sequence of \textit{AtCDEF1} from pEM111 and insert it into a modified pGREEN II vector backbone containing a double 3SS promoter driving transgene expression, yielding the plant expression vector pEM112. The coding sequences of all other genes used to generate transgenic lines were amplified by PCR using Phusion High-Fidelity DNA Polymerase and primers oEM113F x oEM114R (\textit{HtSHINE3}), oEM186F x oEM187R (\textit{AtDEWAX}), oEM198F x oEM199R (\textit{HtCUS1}), oEM220F x oEM221R (\textit{HtMIXTA-like1}). Gibson assembly\textsuperscript{57} was then used to insert the coding sequence of \textit{HtSHN3} into a modified pGREEN II vector backbone containing the promoter region of \textit{AtUBQUITIN10 (PromUBQ10)}, yielding the plant expression vector pEM76; or to insert the coding sequence of \textit{AtDEWAX}, \textit{HtCUS1} or \textit{HtMIXTA-like1} into a modified pGREEN II vector backbone containing a double 3SS promoter, yielding the plant expression vectors pEM98, pEM100 and pEM109, respectively. Gibson assembly was also used to fuse the coding sequence of \textit{HtSHN3} with the sequence coding for the SRDX domain (using primers oEM242F x oEM243R) or with the VP16 domain (using primers oEM168 to oEM171) and produce the plant expression vectors pEM115 and pEM114, respectively (Data S3). The integrity of all plant expression vectors was verified using Sanger sequencing carried out on an Applied Biosystems 3730xl DNA Analyser (Biochemistry DNA Sequencing Facility, University of Cambridge, UK) prior to plant transformation.

Plant transformation

A protocol to generate transgenic \textit{H. trionum} plants was developed using Agrobacterium-mediated transgene delivery followed by tissue culture to induce callus production and plant regeneration.

\textbf{Agrobacterium transformation}

Electrocompetent \textit{Agrobacterium tumefaciens} LBA4404 cells were transformed with plant expression vectors pEM76 (\textit{PromAtUBQ10:HtSHINE3}), pEM98 (35S:\textit{AtDEWAX}), pEM100 (35S: \textit{HtCUS1}), pEM109 (35S: \textit{HtMIXTA-like1}), pEM112 (35S: \textit{AtCDEF1}), pEM114 (35S: \textit{HtSHN3-VP16}) or pEM115 (35S: \textit{HtSHN3-SRDX}). Transformed cells were selected on LB agar plates containing 50 mg.L\textsuperscript{-1} Kanamycin and 25 mg.L\textsuperscript{-1} streptomycin after 48 hours incubation in the dark at 30 °C.

\textbf{H. trionum infection and regeneration}

On day 1, \textit{H. trionum} seeds were sterilised by incubation for 5 min in 70% (v/v) EtOH + 10% (w/v) SDS, followed by 1 min in 95% (v/v) EtOH, and a ddH2O rinse. Dormancy was then disrupted by incubating the seeds for 10 min in 90 °C ddH2O. The sterilised seeds were transferred onto germination media [2.2 g MS salts with vitamins (Duchefa), 25 g sucrose, 6 g Plant agar (Sigma), up to 1 L ddH2O, pH 5.7] and incubated for 36 h in the dark at 30 °C. In parallel, an \textit{Agrobacterium} preculture was started by incubating a colony containing the plant expression vector in 5 mL LB with 50 mg.L\textsuperscript{-1} kanamycin and 25 mg.L\textsuperscript{-1} streptomycin at 28 °C, 220 rpm. Two larger cultures were prepared the following day (day 2), using 50 mL LB supplemented with 50 mg.L\textsuperscript{-1} kanamycin, 25 mg.L\textsuperscript{-1} streptomycin and 50 \textmu L or 500 \textmu L \textit{Agrobacterium} preculture. These cultures were incubated overnight at 28 °C, 220 rpm. On day 3, following overnight incubation, the two cultures were pooled and centrifuged for 10 min at 4,000 rpm. The pellet was re-suspended in 20 mL induction solution [2.2 g MS salts with vitamins (Duchefa), 30 g sucrose, 0.2 mM acetosyringone (Sigma), up to 1 L ddH2O, pH 5.7] and incubated for one hour at 28 °C, 220 rpm. The germinated seeds were dissected under a sterile air hood and the roots, shoot apical meristems and cotyledons were discarded. The hypocotyls were cut into 0.5 cm long sections and incubated with the \textit{Agrobacterium} culture for 10 minutes before rinsing and plating on MS Hib plates [4.4 g MS salts with vitamins (Duchefa), 35 g sucrose, 40 mg.L\textsuperscript{-1} cysteine (Sigma), 15 mg ascorbic acid (Sigma), 8 g Plant agar (Sigma), up to 1 L ddH2O, pH 5.7] containing 0.01 mg.L\textsuperscript{-1} TDZ, 0.05 mg.L\textsuperscript{-1} BA and 0.2 mM acetosyringone (Sigma). Plates were kept in the dark at room temperature for 48 hours. On day 5, hypocotyls were moved to fresh MS Hib plates containing 0.01 mg.L\textsuperscript{-1} TDZ, 0.05 mg.L\textsuperscript{-1} BA, 250 mg.L\textsuperscript{-1} cefotaxime and 150 mg.L\textsuperscript{-1} kanamycin, and plates were transferred to a tissue culture room at 24 °C, 16 hours light, 391 \textmu mol.m\textsuperscript{-2}.s\textsuperscript{-1} illumination. On day 12 and day 19, the hypocotyls were transferred to new MS Hib plates containing 0.2 mg.L\textsuperscript{-1} BA, 250 mg.L\textsuperscript{-1} cefotaxime and 150 mg.L\textsuperscript{-1} kanamycin. From day 33 onwards, calli were moved every 7-10 days to fresh MS Hib plates containing 250 mg.L\textsuperscript{-1} cefotaxime and 150 mg.L\textsuperscript{-1} kanamycin. Calli and regenerating plantlets were assessed weekly under a V12 stereo fluorescence microscope (Zeiss) to check whether the transformation was successful (transformed cells expressing a fluorescent eYFP protein). Once transformed regenerated plantlets were ~1 cm tall, those were cut and transferred to 100 mL sterile Hamilton jars containing 30 mL rooting media [2.2 g MS salts with vitamins (Duchefa), 25 g sucrose, 6 g Plant Agar (Sigma), up to 1 L ddH2O, pH 5.7]. Once roots had developed, regenerated plants were transferred to Levington’s M3 (UK) compost and moved to the greenhouse.

Gene expression analysis

Frozen tissues were ground to a fine powder using mortar and pestle. RNA was extracted using SIGMA Aldrich Spectrum Plant Total RNA kit and retro-transcribed with Invitrogen SuperScript\textsuperscript{TM} following the manufacturer’s instructions. Quantitative real time PCR was performed using a Biorad CFX6 PCR machine and Luna Universal qPCR Master Mix (New England Biolabs). \textit{HtActin} was chosen as a housekeeping gene after testing 5 candidate housekeeping genes across different stages and across biological replicas. The primer efficiencies were 95% for \textit{HtActin}, 100% for \textit{HtSHN3} primers, 105% \textit{HtSHN7}, 104% \textit{HtSHN2}, 97% for \textit{HtCUS1}, 99% for \textit{HtMIXTA}-
like1, 95% for AtCDEF1, 94% for HtGDSL, 98% for HtCYP77. Primer sequences are given in Data S3. The gene expression was calculated relative to the housekeeping gene actin and the common base method, which accounts for the measured efficiency of each primer pair, was used to calculate relative expression levels.** Welch’s t-tests were used to test for the likelihood that transgenic lines and wild type in Figure 4I had the same average expression levels in equivalent petal tissues. RT-PCR was performed by retro-transcribing RNA from leaves of transgenic plants, using SuperScript II (Invitrogen). Manufactured cDNAs were then used as template for the PCR reaction, performed using PCR BIO (PCR Biosystems). The sequences of primers are used in Data S3. 5 μL of the RT-PCR product were collected after 23, 28 and 35 cycles and run on an agarose gel.

Phenotyping
*H. trionum* wild type buds at various stages of development and open flowers from different *Hibiscus* species and *H. trionum* transgenic lines were placed on black velvet and imaged with a Panasonic Lumix DMC-L10 Camera. Dissected petals of *H. trionum* wild type and transgenic lines or from different *Hibiscus* species were imaged either on black velvet with a Panasonic Lumix DMC-L10 Camera (full petal) or on a black stage with a Keyence VHX5000 microscope and a Z1000 lens (close-up imaging).

CryoSEM imaging and Cryo-fractures
The petal surfaces from *H. trionum* wild type or transgenic lines were imaged using a Hitachi S-4700 Cryo-SEM and a Zeiss-Quorum Cryo-SEM. For each sample, about 10 mm² of tissue was mounted on a stage, fixed by cooling in nitrogen slush, before being sputter-coated with gold or platinum in the antechamber of the SEM and introduced into the main chamber to be imaged. Cryo-fractures were performed as described in Airoldi et al.15 with the following modifications: following cryofracture, samples were sputter coated with 8 nm Platinum and imaging was carried out on a Zeiss EVO HD SEM using 6 kV gun voltage and 16 pA probe current.

Cuticle staining and thickness measurements
Stage 3 buds and Stage 5 open flowers were collected from wild type and transgenic lines of *H. trionum* for each solvent, and at least three different petals for each sample type were analysed. For each individual analysis, a droplet of 3 μL of solvent was deposited by a conductive pipette tip at a height of 1.4 mm from the sample plate at the maximum dispensation rate (60 μL/min⁻¹). The liquid junction between the pipette tip and the petal surface was maintained for 30 s for the nonpolar mixture and for 45 s for the polar mixture. The droplet containing the dissolved analytes was then aspirated back into the tip at a height of...
1.2 mm from the sample plate at the maximum aspiration rate (60 µL min⁻¹) and infused in a chip-based nanoESI source (Trivera NanoMate Advion, Ithaca, NY, USA). Samples were sprayed at a gas (N₂) pressure of 0.80 psi at −1.4 kV in negative ionization mode with a transfer capillary temperature of 210 °C. Blanks were analysed by repeating the same procedure on the clean aluminium foil placed underneath the petals, with a dispensation height of 1.2 mm and an aspiration height of 1.0 mm from the surface. Mass spectrometry analysis was performed using an LTQ Velos Orbitrap mass spectrometer (Thermo Scientific, Bremen, Germany) with a resolution of 100 000 at m/z 400 and a typical mass accuracy within ±2 ppm. Data was acquired using an automated acquisition method to record the full scan in the m/z ranges 80–600 and 150–1000. Additional details on the chemical analysis and data processing can be found in Giorio et al.³⁴ and in Zielinski et al.⁵⁵ For the Principal Component Analysis (PCA), compounds that were reliably detected across equivalent samples corresponding either to the smooth or the striated portions of wild type H. trionum petals were selected from the LESA-MS analysis. This yielded a list of 135 compounds that were then used for a target compound search in all other samples (H. trionum transgenic lines and other wild Hibiscus species, Data S4). The ‘ade4’ package⁵⁴ from R⁵² was used to perform the PCAs.

Phylogeny reconstruction
Coding sequence data for Arabidopsis thaliana, Theobroma cacao, Brassica oleracea, Gossypium raimondii and the outgroup Cucumis sativus were downloaded from Ensembl Plants (12/03/2019; release-42). Initial homology was determined using BLASTP⁴⁹ with the settings “-max_target_seqs 200 -evalue 1e-3”. This was conducted on a database containing all amino acid coding sequences from the genomes and the translated HtSHN sequences were used as a query. The corresponding nucleotide sequence for every blast hit was extracted and combined with the HtSHN nucleotide sequences. The sequences were aligned using MAFFT v7.271,⁵⁰ with the settings “–auto –maxiterate 1000”. The alignment was cleaned for a minimum of 10% column occupancy using phyx v.0.999,⑹ and model selection was conducted using the Modelfinder⁵⁹ test as implemented in IQtree v1.6.3.⁵² A maximum likelihood tree was inferred under the general time reversible model, with empirical base frequencies, and a gamma distribution with nine rate categories using IQtree v1.6.3,⁵² with 1000 ultrafast bootstraps⁶⁰ conducted for support.

Mechanical perturbations
As in Airoldi et al.¹⁵ mechanical stress was applied to the petals using an adapted automated confocal micro-extensometer.⁴⁸ The extensometer was constructed using a 50 g force sensor (Futek LSB200), and a Smaract, SLC-1740 - Linear Piezo Stage. To enable visualization of the striations the extensometer was coupled to a Keyence VHX-5000 microscope with a 500-X objective. To avoid dehydration the sample was floated on an isotonic solution of 0.2 M NaCl. Strips of petal were attached to the extensometer using tough-tags (0.94 × 0.50 inches, distal (white), catalogue no. TTSW-1000; DiversifiedBiotech) and cyanoacrylate super glue. The sample was subjected to 60–65 mN of tensile stress. Images were taken of the sample at 200x zoom before and after the experiment.

QUANTIFICATION AND STATISTICAL ANALYSIS
For statistical analysis, Welch’s t-test or ANOVA followed by Tukey’s HSD post-hoc test were used to assess significant differences between the samples using the statistical software R (https://www.r-project.org). The PCA analyses were performed using the ‘ade4’ package⁴ from R.¹⁵ Cell dimensions were quantified using ImageJ (https://imagej.nih.gov/ij/). For each quantification performed, the value of n and what n represents is provided in the method details, the Results and/or the legends of the figures and tables. Following LESA-MS analysis, molecular formulas were assigned using Xcalibur 2.1 (Thermo Scientific, Bremen, Germany) and data were then filtered using a Mathematica 10 (Wolfram Research, UK) code already described in Giorio et al.³⁴