RESEARCH PAPER

Conical petal epidermal cells, regulated by the MYB transcription factor MIXTA, have an ancient origin within the angiosperms

Alison Reed1, Paula J. Rudall2*, Samuel F. Brockington1* and Beverley J. Glover1*,*

1 Department of Plant Sciences, University of Cambridge, Downing Street, Cambridge CB2 3EA, UK
2 Jodrell Laboratory, Royal Botanic Gardens Kew, Richmond, Surrey TW9 3AB, UK

* Correspondence: bjg26@cam.ac.uk

Received 18 February 2022; Editorial decision 9 May 2022; Accepted 20 May 2022

Editor: Madelaine Bartlett, University of Massachusetts Amherst, USA

Abstract

Conical epidermal cells occur on the tepals (perianth organs, typically petals and/or sepals) of the majority of animal-pollinated angiosperms, where they play both visual and tactile roles in pollinator attraction, providing grip to foraging insects, and enhancing colour, temperature, and hydrophobicity. To explore the evolutionary history of conical epidermal cells in angiosperms, we surveyed the tepal epidermis in representative species of the ANA-grade families, the early-diverging successive sister lineages to all other extant angiosperms, and analysed the function of a candidate regulator of cell outgrowth from Cabomba caroliniana (Nymphaeales). We identified conical cells in at least two genera from different families (Austrobaileya and Cabomba). A single SBG9 MYB gene was isolated from C. caroliniana and found to induce strong differentiation of cellular outgrowth, including conical cells, when ectopically expressed in Nicotiana tabacum. Ontogenetic analysis and quantitative reverse transcription–PCR established that CcSBG9A1 is spatially and temporally expressed in a profile which correlates with a role in conical cell development. We conclude that conical or subconical cells on perianth organs are ancient within the angiosperms and most probably develop using a common genetic programme initiated by a SBG9 MYB transcription factor.

Keywords: ANA grade, Cabomba caroliniana, conical cell, MIXTA, Nymphaeales, papillae, petal, tepal.

Introduction

The relationships between flowering plants and their pollinators are key components of ecological networks in almost all terrestrial habitats. Animal pollinators are diverse, with >20 000 pollinating bee species and numerous other insect and vertebrate pollinators (Kevan, 1999). An estimated 35% of global crop production (by volume) depends on biotic pollination, and a decrease in pollinator numbers worldwide has led to a reduction in some crop production rates (Klein et al., 2007). The evolutionary radiation of angiosperms and their insect pollinators has resulted in considerable diversity of floral forms, with a range of floral traits thought to have evolved in response to selective pressure to increase floral attractiveness and memorability (Kevan and Baker, 1983; Schiestl and Johnson, 2013). These traits include flower scent and reward, as well as visual cues involving colour, shape, and patterning.
In the majority of biotically pollinated angiosperm species, the perianth organs involved in visual advertising to pollinators (the tepals or petals) have conical or papillate epidermal cells, at least on the adaxial surface (Kay et al., 1981; Christensen and Hansen, 1998; Ojeda et al., 2009). The observation that conical cells are widespread on petals but rare on leaves indicates that they function to increase floral attractiveness and plant reproductive success, a hypothesis that is supported by field trials in which wild-type flowers of Antirrhinum majus with conical cells received more insect attention and set more fruit than otherwise isogenic flat cells (Glover and Martin, 1998). Conical cell shape not only focuses light into petals, enhancing the pigmented colour (Kay et al., 1998). Conical cells enable insects to minimize energy expenditure by ceasing wing movement and coming to rest while they feed, especially in natural conditions, when flowers are rarely stationary (Rands et al., 2011; Alcorn et al., 2012).

Conical tepal epidermal cells have been described across a phylogenetically broad range of angiosperm species, including many orders of eudicots and monocots (Kay et al., 1981; Christensen and Hansen, 1998; Ojeda et al., 2009; Taneda et al., 2015). In contrast, relatively little is known about their distribution among the ANA-grade lineages (Amborellales, Nymphaeales, Austrobaileyales), which represent separate successional sister lineages to all other extant angiosperms in recent phylogenetic analyses (Fig. 1) (Angiosperm Phylogeny Group, 2009). The seminal investigation of Kay et al. (1981) did not include any ANA-grade families. Despite many morphological studies of flowers of ANA-grade species (e.g. Endress, 2008, 2010), existing descriptions of tepal surfaces are rare, with a few notable exceptions in the waterlily families Nymphaeaceae (Warner et al., 2008, 2009; Zini et al., 2017; Coiro and Barone Lumaga, 2018) and Cabombaceae (Vielette-Guiraud et al., 2011). To address these gaps, we explore the distribution of tepal epidermal cell morphologies across the ANA-grade orders.

The high morphological diversity among the relatively species-poor ANA-grade lineages, coupled with the absence of an extant outgroup closely related to angiosperms, make it difficult to reconstruct hypothetical ancestral states. However, extending information on both comparative morphology and gene function to the ANA-grade lineages is essential in understanding early angiosperm evolution. To explore these aspects, we analysed MIXTA-like gene function from an ANA-grade MIXTA AmMYBML1, 2, and 3, with overlapping but non-redundant functions (Perez-Rodriguez et al., 2005; Baumann et al., 2007; Jaffé et al., 2007).

Phylogenetic analysis of SBG9 R2R3 MYB genes has revealed an ancient duplication that occurred before the origin of seed plants, resulting in two strongly supported clades: SBG9A, which encompasses AmMIXTA and AmMYBML1, 2, and 3, and SBG9B (Brockington et al., 2013). SBG9A MYB genes have now been functionally characterized from the basal eudicot Thalictrum thalictroides (Di Stilio et al., 2009), the monocot Dendrobium crumenatum (Gilding and Marks, 2010), and a range of eudicot species (Baumann et al., 2007; Machado et al., 2009; Gilding and Marks, 2010; Brockington et al., 2013). To date, the only SBG9B gene that has been characterized is MYB17-like from Lotus japonicus (Brockington et al., 2013). All SBG9 MYB transcription factors analysed so far play a role in epidermal cell outgrowth, often associated with petal conical cells.

The morphological data that we present here, exploring conical tepal epidermal cell distribution across the ANA-grade angiosperms, combined with our analysis of SBG9 MYB function in C. caroliniana, suggest that conical petal epidermal cells, and the anisotropic cell expansion that underpins their development in eudicots, are an ancestral feature of flowering plants.
Materials and methods

Sources of plant material

Materials of ANA-grade angiosperms examined for morphology are indicated in Table 1.

All C. caroliniana flowers and vegetative tissues for RNA/DNA extraction were purchased online from Plants Alive Ltd (Stone, Staffordshire, UK) and grown in a glass aquarium in 10 × 3.5 cm round pots with rockwool, weighted down using lead strips.

Material preparation and preservation

Flowers and inflorescences were harvested and immediately fixed in FAA [60% ethanol (EtOH); 6% formaldehyde; 5% acetic acid]. The flowers were left in fixative for 72 h and then transferred to 70% EtOH solution. Where it was impractical to collect fresh material, dental wax (Elite HD vinylpolysiloxane) was used to make a high resolution mould of plant surfaces, and accurate replicas were produced using Devcon 2 Ton epoxy resin.

Scanning electron microscopy

Prior to examination using SEM, fixed samples (stored in 70% EtOH) were dehydrated in a series of ascending EtOH concentrations and critical point dried in an Autosamdrí 815B critical point drier. Samples were sputter-coated in platinum using an Emitech K550 sputter coater. The coated specimens were viewed using a Hitachi FE-SEM S-4700 scanning electron microscope (Hitachi Hi-Tec Technologies, Maidenhead, UK) and images captured using PCI software (Quartz Imaging Corp., Vancouver, Canada).

Epoxycast material was coated in gold or chromium using a Quorum K756X sputter coater. Samples were then viewed using a FEI Philips XL30 FEGSEM scanning electron microscope 0.5–30 KeV with an Oxford Instruments INCA EDX system running a 30 mm² SiLi thin window pentafet EDX detector.

Isolation of CcSBG9A1

RNA was extracted from floral tissue frozen in liquid nitrogen, using a cetyltrimethylammonium bromide (CTAB)-based extraction followed by chloroform extraction and precipitation in 4 M LiCl. Prior to cDNA synthesis, RNA samples were treated with DNase I. cDNA was synthesized from total RNA using Bioline Bioscript™ reverse transcriptase, and amplified with 100% ethanol between each tissue and flower. RNA for qPCR was extracted using Plant RNA Reagent (Invitrogen™), treated with AmpliPrep™ DNA-free™ and converted to cDNA using Invitrogen Superscript III, primed using oligo(dT)20, and a random hexamer.

SBG9 R2R3 MYB-like sequences from ANA-grade angiosperms (Amborella trichopoda and Nuphar sp.) were obtained from the genomics database of the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/). Sequences were aligned using Se-Al v2.0a11 and used to design degenerate primers. RACE was used to amplify full-length cDNAs which were cloned into pGEM-T for sequencing and further analysis. Primer sequences are listed in Supplementary Table S1.

Phylogenetic analysis

The putative Cabomba SBG9A protein was analysed in the context of previously published alignments generated for the SBG9A clade (Brockington et al., 2013). The sequence was aligned using the translation alignment function of nucleotide sequences in Geneious, and subject to a FastTree algorithm, using the GTR model. SH support values were generated during the FastTree analysis, and reported on the tree topology. All branches with <0.50 SH support were removed.

Ectopic expression in Nicotiana tabacum

The binary vector pGREENII0029:35S with the LaaZ gene removed and replaced with a double copy of the Cauliflower mosaic virus (CaMV) 35S gene promoter (Hellens et al., 2000) was used for gene transfer. CcSBG9A1 was inserted as an EcoRI fragment from pGEM-T into pGEM. The binary vector was transferred into Agrobacterium tumefaciens GV3101 by electroporation. Transformation of N. tabacum var. Samsun was conducted using a modified version of the leaf disk protocol of Horsch et al. (1985). Transgenic plants were grown to maturity in a controlled greenhouse environment at 26 °C with a 16 h light regime, and transgene insertion and expression were confirmed using PCR with genomic DNA and reverse transcription–PCR (RT–PCR) with leaf RNA (see Supplementary Fig. S1).

Quantitative RT–PCR (qPCR)

Mature flowers and buds were dissected on three independent plants using micro-dissecting forceps. Carpels, stamens, and tepals were removed separately and immediately frozen in liquid nitrogen. Forceps were cleaned with 100% ethanol between each tissue and flower. RNA for qPCR was extracted using Plant RNA Reagent (Invitrogen™), treated with Ambion TURBO DNA-free™, and converted to cDNA using Invitrogen Superscript III, primed using oligo(dT)20, and a random hexamer.

Table 1. Species and material examined

| Family                  | Species examined                        | Material examined          |
|-------------------------|-----------------------------------------|----------------------------|
| Amborellaceae           | Amborella trichopoda Bailey.            | HK: s.n.                   |
| Nymphaeaceae           | N. odorata Alton subsp. Tuberosa Wiersema & Hellq. | HK: 1969-19765             |
|                         | N. violacea Leh.                       | HK: 2007-1810              |
|                         | Victoria cruziana Orbign.              | HK: 2011-1436              |
| Cabombaceae            | C. caroliniana A.Gray                  | HK: s.n. and commercial source (living plants) |
|                         | C. furcata Schult. & Schult.f          | Commercial source (living plants) |
| Austrobaileyaceae      | Austrobaileyá scandens C.T.White        | HK: 2012-464               |
| Schisandraceae         | Kadsura heterocitha Craib.             | HK: 1985-4488              |
|                         | K. japonica Benth.                     | HK: 1989-3952              |
|                         | Schisandra rubriflora Rehder & E.H.Wilson | HK: 1969-19804             |
| Iliciaceae             | Ilicium floridanum J.Ellis              | HK: 2011-880               |
|                         | I. simonsii Maxim.                     | HK: 1994-3682              |
| Trimeniaceae           | Trimenia moorei (Oliv.) Philipson       | s.n.                       |

HK indicates material cultivated at RBG Kew; s.n. indicates that the accession number is absent.
Conical or subconical tepal epidermal cells occur in several ANA-grade species

A summary of tepal surfaces of ANA-grade species is given in Table 2 and Fig. 2, with emphasis on the distribution of conical cells and surface patterning. Following the terminology outlined by Kay et al. (1981), conical cells and papillate cells are more or less synonymous; they protrude significantly outwards from the epidermis and have a distinct tip or peak (subconical cells slightly less so). Lenticular cells are only slightly domed and lack a distinct tip. Flat cells show no clear sign of protrusion. In surface view, cell shape ranges from rounded to elongated, often on the same petal, with elongated cells mostly occurring at the petal/tepal base. In transverse section, cell shape ranges from flat through domed/lenticular to conical; as noted above, the presence of a distinct tip or peak separates conical cells from domed cells. Fine details of surface sculpturing range from smooth to striate.

ANA-grade taxa display a diverse tepal surface structure, consistent with their diverse floral morphology. Of the seven ANA-grade families, distinctly conical epidermal cells are present in species of Austrobaileya (Austrobaileyaceae), Cabomba (Cabombaceae), and the staminoidal tepals of Victoria cruziana (Nymphaeaceae). In Austrobaileya scandens, conical cells cover most parts of the flower (tepals, stamens, and staminodia) except the carpels and the central regions of the outer tepals; all cells and papillae possess fine radiating striations. The adaxial tepal surface of A. scandens is complex, with large stomata and secretory cells also present; stomata are more abundant in central flat-celled regions that lack conical papillae. In Cabomba, non-striated conical cells cover the adaxial tepal surfaces of anthetic flowers, especially towards the tepal apex, with relatively flat surfaces at the tepal bases. In V. cruziana, only the innermost tepals and staminoid tepals have conical cells, which are often striated.

Subconical or deeply domed cells are present in Amborella (Amborellaceae), Illicium (Illiciaceae), and Kadsura (Schisandraceae). In A. trichopoda, the tepals are thick and reflexed, with a central adaxial groove surrounded by bulbous regions. The adaxial tepal surfaces display diverse morphology, though epidermal cells are mostly deeply domed, often flat-topped or angular with chaotic fine surface patterning. In Illicium simmonsii, tepal surfaces range from flat-celled to domed, sometimes with a central prominence and always with striations. In Kadsura heteroclitica, most of the tepal surface is covered by subconical or occasionally conical cells. Conical and subconical cells are absent from Hydatellaceae, most Nymphaeaceae, Schisandra (Schisandraceae), and Trimeniaceae.

**Results**

**Conical or subconical tepal epidermal cells occur in several ANA-grade species**

CcActin was selected as a reference gene based on successful preliminary trials demonstrating stable expression across tissues. Primer sequences are provided in Supplementary Table S1. Forty cycles of PCR were performed using either a BioRad DNA Engine Thermocycler or a CFX Connect Real-Time PCR Detections System (185-5200). A melting curve was performed from 60 °C to 95 °C with readings taken at 0.5 °C intervals. Relative gene expression was quantified using an Opticon Monitor 3 and CFX Manager software (both BioRad Laboratories, Inc.). Ct values were exported to Microsoft Excel, and ΔCt values were calculated by subtracting the Ct of the reference gene, actin. Each dataset was statistically analysed in Excel using a t-test.

**Sequence and phylogenetic placement of CcSBG9A1**

To determine whether the anisotropic outgrowth of conical cells of ANA-grade tepals is regulated by the same R2R3MYB transcription factors (subgroup 9 MIXTA-like proteins) that control conical petal cell development in angiosperms, we isolated a SBG9A R2R3 MYB gene from C. caroliniana using degenerate PCR. The predicted protein contains the amino acid motif that is characteristic of the SBG9A lineage, which includes the well-characterized MIXTA and MIXTA-like genes from eudicots (Brockington et al., 2013). The CcSBG9A1 protein shows a high degree of similarity with SBG9A MYB proteins from other ANA-grade genera. One notable exception is the occurrence of an amino acid substitution (lysine in place of threonine) at the centre of the highly conserved SBG9A motif. Phylogenetic analysis of SBG9A MYB genes, with the inclusion of the CcSBG9A1 gene isolated here, confirms that CcSBG9A1 groups with SBG9A MYB genes from other ANA-grade genera (Nuphar and Amborella) (Fig. 3; Supplementary Fig. S2). Together with monocot sequences and early diverging eudicot sequences, these ANA-grade sequences diverged before the main duplication within the core eudicots that gave rise to the MIXTA and MIXTA-like clades (Brockington et al., 2013). While there are recent, lineage-specific duplications of this gene family within A. trichopoda and Nuphar advena, there is no evidence from this analysis of a deep duplication event within the ANA grade. Since our degenerate PCR identified no other gene fragments, there is only a single SBG9A EST from Cabomba aquatica, and our phylogenetic analysis provides no evidence of a deep duplication event, we tentatively conclude that there is only a single representative of MYB SBG9A in the Cabomba genome.

**Transgenic analysis of CcSBG9A1 function in a tobacco bioassay**

To explore the ability of the CcSBG9A1 protein to induce anisotropic cell expansion and cellular outgrowth, we generated nine independent transgenic lines of tobacco (N. tabacum var. Samsun) expressing the gene from the double CaMV35S promoter (Supplementary Fig. S1). The same bioassay has been used to explore the function of eudicot members of this gene family with different genes able to induce cellular outgrowth on different subsets of tobacco organs (Glover et al., 1998; Perez-Rodriguez et al., 2005; Baumann et al., 2007; Jaffé et al., 2007; Brockington et al., 2013). The transgenic plants displayed
a reduction in flower colour, the transgenic flowers appearing a much paler shade of pink relative to wild-type lines (Fig. 4A). The anthers of several of these lines—those also showing the strongest change in epidermal phenotype—failed to fully dehisce. These phenotypic outcomes have been described in other studies expressing SBG9A MYB genes in tobacco (Glover et al., 1998).

Previous studies have reported that the ectopic expression of SBG9A MYB genes in tobacco most commonly induces cell outgrowth on the ovary epidermis. In wild-type plants, epidermal cells of the ovary have a rounded base shape and are flat or slightly lenticular (Fig. 4B). In all lines of transgenic tobacco expressing 35S:CcSBG9A1, ectopic cell outgrowths were present on the surface of the ovary (Fig. 4C). The majority of epidermal cells had an altered appearance, and conical cells and trichomes were present in approximately equal abundance. These cell protrusions ranged from 10 µm to 350 µm in length. In wild-type tobacco flowers, the style and stamen filaments have uniformly flat elongate cells (Fig. 4D). In transgenic lines expressing 35S:CcSBG9A1, ectopic cell protrusions were observed on both floral organs, although they were less dense than on the ovary. On the style of plants with a strong phenotype, protrusions ranged from conical cells to long-stalked trichomes (Fig. 4E).
filament of transgenic flowers, short trichomes <30 µm in length were the most common type of protrusion. The anther of wild-type flowers has a regular arrangement of conical cells (Fig. 4F). In all transgenic lines, epidermal cells had an altered shape and distinct bulbous tip (Fig. 4G). For some cells, the tips of cells were extended into trichomes of varying lengths.

At the tip of the corolla, the epidermis of wild-type flowers has a regular arrangement of conical cells with a pronounced bulb at the tip of each cell (Fig. 4H). In all lines expressing...
Fig. 3. Maximum likelihood phylogram of SGB9A MYB genes from seed plants. SH support values are reported on the tree topology. The MIXTA and MIXTA-like clades of eudicot family members are marked. CcSGB9A1 is highlighted in red.
Conical petal epidermal cells | Page 5497 of 5502

35S::GcSBG9A1, epidermal cell shape was more variable particularly at the tips of cells, which protruded to varying degrees (Fig. 4I). Multicellular trichomes >50 µm in length, and sometimes glandular, were found to be sparsely distributed amongst the conical cells.

On the adaxial epidermis of wild-type leaves, cells are largely flat with a rounded base shape. Long multicellular trichomes and shorter hyathode-type trichomes are irregularly distributed on the leaf epidermis (Fig. 4I). In several transgenic lines, some of the long multicellular trichomes had multiple branches (Fig. 4K). Between these trichomes, the epidermal cells remained largely flat or lenticular, but many of these cells developed a distinct peak or tip (Fig. 4K). Occasionally, these cells also had an altered overall shape and were distinctly conical. No changes in epidermal morphology were observed on the abaxial leaf epidermis of transgenic lines.

Ontogeny of tepal epidermal outgrowth in Cabomba caroliniana

Tepal epidermal morphology was characterized at five developmental stages of *C. caroliniana*: (1) 1 mm buds; (2) 2 mm buds; (3) 4 mm buds; (4) 5 mm buds; and (5) 7 mm buds or flowers at anthesis. Flowers of *Cabomba* have whorled floral phyllotaxy, with two whorls of three petaloid tepals forming in alternating positions (Fig. 5A). The inner tepals are developmentally retarded with respect to the outer tepals and other floral organs, and thus for each stage the tepals from the inner and outer whorls were imaged separately (Vialette-Guiraud et al., 2011). Specific zones along the length of the tepal were identified for comparative analysis, as outlined in Fig. 5A.

At the youngest stage (stage 1), the inner and outer whorls of tepals were indistinguishable, and there was no evidence of cell outgrowth (Fig. 5B). By stage 2, nectaries are present towards the base of the inner tepals, although these are restricted to the very outer edges of the tepal (Fig. 5C). Cells at the tip of the tepal are similar in appearance in inner and outer tepal whorls (Fig. 5D), while those at the base of the tepal are more variable. There was no evidence of cell outgrowth. By stage 3, pronounced conical cells are visible at the tip of both the inner and outer tepals. On the inner tepals, these cones have a more pointed shape (Fig. 5E), while they are distinctly rounded on the outer whorl. At the base of the inner tepal, nectaries are well developed (Fig. 5F). Stage 4 shares an almost identical phenotype with stage 3 (Fig. 5G, H). By stage 5, conical epidermal cells at the tips of the tepals are more uniform, but there is no change in their total degree of protrusion (Fig. 5I, J).

Expression analyses of CcSBG9A1 in Cabomba caroliniana

qPCR was used to determine whether expression levels of *C. SBG9A1* correlated with conical cell development in *C. caroliniana*. Expression analyses were conducted across a range of floral tissues—tepals (pooled inner and outer whorls), stamens, and carpels—and three developmental stages [pooled bud stages 1 and 2 (<3 mm), pooled bud stages 3 and 4 (3–5 mm in length), and bud stage 5 (>5 mm in length)]. Mean expression values for each tissue and developmental stage were calculated relative to expression levels of *CaActin* from three technical and three biological replicates (Fig. 5K). The highest level of *CcSBG9A1* expression was in young tepals at stages 1 and 2, immediately prior to the appearance of conical cells (at stage 3). There were very low levels of *CcSBG9A1* expression in tepals larger than 3 mm and in mature flowers (>5 mm). A t-test confirmed that *CcSBG9A1* expression in the tepals of <3 mm buds is significantly higher than in the tepals of >5 mm buds [t(4)=5.92, P<0.01]. When *CcSBG9A1* expression is compared in the different tissues of <3 mm buds, it is significantly higher in the tepals relative to the stamens [t(4)=6.37, P<0.01] or carpels [t(4)=6.12, P<0.01].

Discussion

Conical petal epidermal cells are present in several ANA-grade angiosperms

Our examination of species from the three ANA-grade orders (Fig. 2) reveals complex tepal surfaces in ANA-grade species, consistent with the diversity of flower structure in these taxa which contributes to the bigger picture of perianth epidermal morphology evolution across the angiosperms. Tepal surfaces are rarely entirely uniform, and can differ on the same flower and even on the same tepal. Two ANA-grade genera, *Cabomba* and *Austrobaileya*, possess distinctly conical cells over most of the adaxial tepal surface. In several other ANA-grade genera (e.g. *Kadsura* and *Victoria*), cells are conical or subconical on some parts of the tepal surface. *Amborella trichopoda*, the putative sister to all other angiosperms, possesses strongly domed cells. In contrast, a few ANA-grade species possess mostly flat cells on the tepal surface (e.g. *Nuphar* and *Trimenia*).

The conical-papillate petal epidermis represents the most common type in angiosperms (Kay et al., 1981), but there also exist many subconical types with a rounded or flattened apex, as we have found in *Amborella* and *Kadsura*. The widespread distribution of conical or subconical cells in all three ANA-grade lineages indicates that the capacity to produce them is of ancient origin. Few studies have examined the apparently simple transition from a lenticular or subconical cell to a conical cell. In many eudicots, formation of both conical cells and trichomes on the petal epidermis is determined by SBG9A MYB transcription factors (Martin et al., 2002; Pérez-Rodríguez et al., 2005). Ectopic expression of SBG9A MYB genes can induce both conical and lenticular cellular outgrowth (Martin et al., 2002; Jaffé et al., 2007). In some eudicots, there is clear evidence for an evolutionary loss of the conical cell form within a specific taxonomic group or natural community (e.g. Ojeda et al., 2009, 2016).

Expression analyses of CcSBG9A1 in Cabomba caroliniana

qPCR was used to determine whether expression levels of *C. SBG9A1* correlated with conical cell development in *C. caroliniana*. Expression analyses were conducted across a range of floral tissues—tepals (pooled inner and outer whorls), stamens, and carpels—and three developmental stages [pooled bud stages 1 and 2 (<3 mm), pooled bud stages 3 and 4 (3–5 mm in length), and bud stage 5 (>5 mm in length)]. Mean expression values for each tissue and developmental stage were calculated relative to expression levels of *CaActin* from three technical and three biological replicates (Fig. 5K). The highest level of *CcSBG9A1* expression was in young tepals at stages 1 and 2, immediately prior to the appearance of conical cells (at stage 3). There were very low levels of *CcSBG9A1* expression in tepals larger than 3 mm and in mature flowers (>5 mm). A t-test confirmed that *CcSBG9A1* expression in the tepals of <3 mm buds is significantly higher than in the tepals of >5 mm buds [t(4)=5.92, P<0.01]. When *CcSBG9A1* expression is compared in the different tissues of <3 mm buds, it is significantly higher in the tepals relative to the stamens [t(4)=6.37, P<0.01] or carpels [t(4)=6.12, P<0.01].

Discussion

Conical petal epidermal cells are present in several ANA-grade angiosperms

Our examination of species from the three ANA-grade orders (Fig. 2) reveals complex tepal surfaces in ANA-grade species, consistent with the diversity of flower structure in these taxa which contributes to the bigger picture of perianth epidermal morphology evolution across the angiosperms. Tepal surfaces are rarely entirely uniform, and can differ on the same flower and even on the same tepal. Two ANA-grade genera, *Cabomba* and *Austrobaileya*, possess distinctly conical cells over most of the adaxial tepal surface. In several other ANA-grade genera (e.g. *Kadsura* and *Victoria*), cells are conical or subconical on some parts of the tepal surface. *Amborella trichopoda*, the putative sister to all other angiosperms, possesses strongly domed cells. In contrast, a few ANA-grade species possess mostly flat cells on the tepal surface (e.g. *Nuphar* and *Trimenia*).

The conical-papillate petal epidermis represents the most common type in angiosperms (Kay et al., 1981), but there also exist many subconical types with a rounded or flattened apex, as we have found in *Amborella* and *Kadsura*. The widespread distribution of conical or subconical cells in all three ANA-grade lineages indicates that the capacity to produce them is of ancient origin. Few studies have examined the apparently simple transition from a lenticular or subconical cell to a conical cell. In many eudicots, formation of both conical cells and trichomes on the petal epidermis is determined by SBG9A MYB transcription factors (Martin et al., 2002; Pérez-Rodríguez et al., 2005). Ectopic expression of SBG9A MYB genes can induce both conical and lenticular cellular outgrowth (Martin et al., 2002; Jaffé et al., 2007). In some eudicots, there is clear evidence for an evolutionary loss of the conical cell form within a specific taxonomic group or natural community (e.g. Ojeda et al., 2009, 2016).
Fig. 4. Ectopic expression of CcSBG9A1 in tobacco. (A) Wild-type (left) and transgenic (centre, right) flowers. (B) SEM image of wild-type tobacco carpel epidermis. (C) SEM image of tobacco carpel expressing CcSBG9A1. (D) SEM image of wild-type tobacco style epidermis. (E) SEM image of tobacco style expressing CcSBG9A1. (F) SEM image of wild-type tobacco anther head. (G) SEM images of anther heads of two independent tobacco lines expressing CcSBG9A1. (H) SEM image of wild-type tobacco petal epidermis. (I) SEM images of petals of two independent tobacco lines expressing CcSBG9A1. (J) SEM image of wild-type tobacco leaf adaxial epidermis. (K) SEM images of adaxial leaf epidermis of two independent tobacco lines expressing CcSBG9A1. All scale bars=50 μm.
Fig. 5. Development of conical tepal epidermal cells in *Cabomba caroliniana*. (A) Schematic diagram showing the tepal morphology of *Cabomba* as used for the SEM developmental series. Carpels and stamens are not shown. The positions of the inner and outer tepal whorls (see key), nectaries, and sampling zones (1–3) are marked. (B) SEM image of adaxial tepal epidermis at stage 1, zone 3. Inner and outer tepals are indistinguishable at this stage. (C) SEM image of adaxial epidermis of inner tepal at stage 2, zone 3. (D) SEM image of adaxial epidermis of outer tepal at stage 2, zone 1. (E) SEM image of adaxial epidermis of inner tepal at stage 3, zone 1. (F) SEM image of adaxial epidermis of outer tepal at stage 3, zone 3. (G) SEM image of adaxial epidermis of inner tepal at stage 4, zone 1. (H) SEM image of adaxial epidermis of outer tepal at stage 4, zone 1. (I) SEM image of adaxial epidermis of inner tepal at stage 5, zone 1. (J) SEM image of adaxial epidermis of outer tepal at stage 5, zone 1. (K) qPCR analysis of CcSBG9A-1 expression in different tissues and at different developmental stages (<3 mm=stages 1 + 2; 3–5 mm=stages 3 + 4; >5 mm=stage 5) of *Cabomba caroliniana*. Target gene expression was quantified relative to actin. Values represent mean expression values and SEs ($n=3$). All scale bars=20 µm.
Pollination biology is also diverse among ANA-grade species, though data are relatively sparse for some taxa (Thien et al., 2009; Endress, 2010; Luo et al., 2018). Beetle pollination is common in the waterlily family Nymphaeaceae and in some Schisandraceae; flies are the major pollinators of *Austrobailey a* and *Illicium*; and Schisandraceae are predominantly pollinated by nocturnal gall midges (Endress, 2010; Luo et al., 2018). Petal surfaces with domed and/or conical cells are frequently involved in scent production (Vogel, 1990). The tiny white flowers of *Amborella* produce a scent that attracts nocturnal moths and other insects (Thien et al., 2009). A likely source for the scent is the prominent regions of the central part of the tepal surface, which function as osmophores. The waterlily genus *Cabomba*, which possesses prominent conical cells, is unusual among ANA-grade angiosperms in possessing well-defined nectaries on the surfaces of the inner tepals; the nectar provides a reward to visiting pollinating insects such as bees, wasps, and flies (Schneider and Jeter, 1982; Taylor and Williams, 2009; Vialette-Guiraud et al., 2011; Luo et al., 2018). Two genera that lack conical cells are probably abiotically pollinated: *Trithuria* (Hydatellaceae) and *Braesia* (Cabombaceae), supporting a correlation between conical cells and pollinator attraction.

An SBG9A MYB transcription factor from an early diverging angiosperm can induce ectopic conical cell development

To analyse the homologies of conical tepal epidermal cells, we explored the developmental genetic processes underpinning cellular differentiation. A common developmental programme could suggest a single ancestral origin followed by repeated evolutionary losses or modifications. The SBG9A MYB transcription factors are known to control petal epidermal cell outgrowth in both eudicots (Noda et al., 1994; Machado et al., 2009; Di Stilio et al., 2009; Brockington et al., 2013) and monocots (Gilding and Marks, 2010). We therefore examined whether this subgroup of MYB genes could perform similar functions in ANA-grade angiosperms, suggesting a single origin of conical cells.

Analyses of SBG9A MYB protein function are sometimes hampered by the many duplications seen within the gene family at different phylogenetic levels (Bedon et al., 2014). However, our phylogenetic reconstruction, coupled with evidence from published transcriptomes, demonstrates only a single SBG9A gene in the *Cabomba* genome. The gene family is divided into MIXTA and MIXTA-like clades following a duplication at the base of the eudicots (Brockington et al., 2013), but the ANA-grade members form a clade that diverged before this duplication (Fig. 2). Furthermore, although there are lineage-specific duplications in some genera within this clade, we found no evidence for a deep duplication event within the ANA-grade lineages.

Ectopic expression of *GcSBG9A1* in tobacco revealed that the protein has the ability to induce anisotropic cell expansion and cellular outgrowth in all tissues tested, indicating that it is able to induce cellular differentiation alone (or with a ubiquitously expressed partner). The strength of the ectopic expression phenotype is remarkable. The same heterologous approach using the same strong constitutive promoter (a double copy of the 35S promoter from CaMV) in *N. tabacum* has been used for several other angiosperm SBG9A MYB genes over the last 20 years. This list includes *TmMYBML2* from the basal eudicot *Thalictrum thaloides* (Di Stilio et al., 2009), *AtMYB16* and *AtMYB106* from Arabidopsis (Baumann et al., 2007; Gilding and Marks, 2010), *PhMYB1* from *Petunia hybrida* (Baumann et al., 2007), and the four SBG9-A genes in *A. majus* (AmMIXTA, *AmMIXTA-LIKE 1*, *AmMIXTA-LIKE 2*, and *AmMIXTA-LIKE 3*) (Glover et al., 1998; Perez-Rodriguez et al., 2005; Baumann et al., 2007; Jaffé et al., 2007). It is notable that *GcSBG9A1* induces a much stronger phenotype than most previously characterized SBG9A MYB genes in this bioassay. For example, *PhMYB1*, *AmMYBML2*, *AtMYB16*, and *TmMYBML2* share similar expression patterns and similar phenotypes when ectopically expressed in tobacco. Transgenic tobacco plants exhibit ectopic outgrowths on the surface of the ovary, as well as an increase in the height and change in shape of conical cells on the corolla. However, these outgrowths never develop into multicellular trichomes, and no changes were observed to the other floral organs, or vegetative leaves (some changes were observed on inflorescence leaves) (Baumann et al., 2007; Di Stilio et al., 2009). The strongest reported phenotypes from this bioassay are for *N. tabacum* plants overexpressing *AmMIXTA*, which exhibit long multicellular trichomes on the ovary and at the tip of the inner corolla. On the leaves, several parallels can be drawn with the effects of *GcSBG9A1* expression. For example, the majority of cells on the adaxial leaf epidermis have a single, central outgrowth. These outgrowths are almost identical on the adaxial leaf epidermis of transgenic tobacco expressing 35S:*GcSBG9A1* and 35S:*AmMIXTA*. Long-stalked, multicellular branched trichomes were also observed on the adaxial leaf epidermis of both transgenic lines (Glover et al., 1998; Perez-Rodriguez et al., 2005). Phenotypic strength may be affected by the position of transgene insertion and the transgene expression level, so our conclusions here must be tentative, but it is nonetheless notable that the phenotypes observed in this study are consistently stronger than those for most related genes using the same bioassay system.

The four SBG9A genes in *A. majus* have arisen from Antirrhineae-specific duplication events within the MIXTA (*AmMIXTA* and *AmMIXTA-LIKE 1*) and MIXTA-like (*AmMIXTA-LIKE 2* and *AmMIXTA-LIKE 3*) clades. These four genes show sequence homology and may have overlapping functions, but they are not functionally redundant. It has been suggested that formation of fully developed conical cells on the petals of *A. majus* requires two distinct activities. For example, *AmMIXTA* and *AmMYBML1* may be responsible for initiating conical cell development, while *AmMYBML2* and *AmMYBML3* coordinate a second stage of elongation that leads to a complete cone (Perez-Rodriguez et al., 2005).
Our study shows that the gene duplication event that led to formation of the MIXTA and MIXTA-like clades, as well as the Antirrhineae-specific duplication event that gave rise to AmMYBML1, 2, and 3, arose after the divergence of Cabomba (Fig. 2). There is no evidence of an ancient gene duplication event in the MIXTA and MIXTA-like clades within either the early diverging angiosperm or the early land plant lineages. In turn, we infer that the single CcSBG9A1 protein plays a crucial role in inducing anisotropic cell expansion and coordinating conical cell development in C. caroliniana. The ability of CcSBG9A1 to induce the formation of ectopic cell outgrowths in tobacco indicates that the encoded CcSBG9A1 protein is capable of regulating transcriptional targets similar to other members of the SBG9A lineage. The strength of the phenotype suggests that CcSBG9A1 is a particularly effective transcriptional regulator of the downstream cellular differentiation pathway and has the potential to act as a master regulator of epidermal cell outgrowth.

A SBG9A MYB transcription factor is expressed specifically in developing tepals of Cabomba caroliniana, immediately prior to conical cell outgrowth

Since it is not possible to transform C. caroliniana, we sought additional correlative evidence in support of a role for CcSBG9A1 in conical cell development. Although CcSBG9A1 is clearly able to induce cellular outgrowth, its native phenotypic effects will depend on the transcriptional profile of the gene encoding it. We used an ontogenetic series to determine that epidermal cell outgrowth in the Cabomba tepal occurs at growth stage 3, when buds are between 2 mm and 4 mm long. We predicted that the transcriptional regulator controlling cellular outgrowth would be expressed in earlier stages of tepal development. qPCR analyses of dissected tissues revealed that CcSBG9A1 is most strongly expressed in tepals of buds <3 mm in length. Transcript is almost undetectable in other floral organs (stamens and carpels) and in tepals at later developmental stages when the conical cells are already present. This expression pattern correlates strongly with a role in regulating conical tepal epidermal cell development.

Conclusions

Our study clearly demonstrates the presence of conical perianth epidermal cells in some of the earliest surviving angiosperm lineages. Our combined strong, if correlative, evidence suggests that outgrowth of the conical cells in Cabomba is regulated by the same MYB SBG9A-initiated pathway that regulates petal cell development in eudicots. This ancient origin for conical cells and their developmental programme suggest that the many angiosperm species that lack conical petal cells represent secondary losses of an ancestral character. We hypothesize that changes in cis regulation or protein function of SBG9 MYB genes, potentially correlated with shifts in pol-
of the MIXTA gene family highlights potential targets for the study of cellular differentiation. Molecular Biology and Evolution 30, 526–540.

Christensen KI, Hansen HV. 1998. SEM-studies of epidermal patterns of petals in the angiosperms. Copenhagen: Council for Nordic Publications in Botany.

Coiro M, Barone Lumaga MR. 2018. Disentangling historical signal and pollinator selection on the micromorphology of flowers: an example from the floral epimorph of Nymphaeaceae. Plant Biology 20, 902–915.

Di Stilio VS, Martin C, Schulfer AF, Connelly CF. 2009. An ortholog of MIXTA-like2 controls epidermal cell shape in flowers of Thalictrum. New Phytologist 178, 718–728.

Dubos C, Stracke R, Grotewold E, Weisshaar B, Martin C, Lepiniec L. 2010. MYB transcription factors in Arabidopsis. Trends in Plant Science 15, 573–581.

Dyer AG, Whitney HM, Arnold SEJ, Glover BJ, Chittka L. 2007. Mutations perturbing petal cell shape and anthocyanin synthesis influence bumblebee perception of Antirrhinum major flower colour. Arthropod-Plant Interactions 1, 45–55.

Endress PK. 2008. Perianth biology in the basal grade of extant angiosperms. International Journal of Plant Science 169, 844–862.

Endress PK. 2010. The evolution of floral biology in basal angiosperms. Philosophical Transactions of the Royal Society B: Biological Sciences 365, 411–421.

Gilding EK, Marks MD. 2010. Analysis of purified glabra3-shapeshift trichomes reveals a role for NOECK in regulating early trichome morphogenetic events. The Plant Journal 64, 304–317.

Glover BJ, Martin C. 1998. The role of petal cell shape and pigmentation in pollination success in Antirrhinum majus. Heredity 80, 778–784.

Glover BJ, Perez-Rodriguez M, Martin C. 1998. Development of several epidermal cell types can be specified by the same MYB-related plant transcription factor. Development 125, 3497–3508.

Gorton HL, Vogelmann TC. 1996. Effects of epidermal cell shape and pigmentation on optical properties of Antirrhinum petals at visible and ultra-violet wavelengths. Plant Physiology 11, 879–889.

Hellers RP, Edwards EA, Leyland NR, Bean S, Mullineaux PM. 2000. pGreen: a versatile and flexible binary Ti-vector for Agrobacterium-mediated plant transformation. Plant Molecular Biology 42, 819–832.

Horsch RB, Fry JE, Hoffman NL, Eichholtz D, Rogers SG, Fraley R, Schiestl FP, Johnson SD. 1983. Insects as flower visitors and pollinators. Annual Review of Entomology 28, 407–453.

Jaffé FW, Tattersall A, Glover BJ. 2007. A truncated MYB transcription factor from Antirrhinum majus regulates epidermal cell outgrowth. Journal of Experimental Botany 58, 1515–1524.

Kay OON, Daoud HS, Stirton CH. 1981. Pigment distribution, light reflection and cell structure in petals. Botanical Journal of the Linnean Society 83, 57–83.

Kevan PG. 1999. Pollinators as bioindicators of the state of the environment: species, activity and diversity. Agriculture Ecosystems and Environment 74, 373–393.

Kevan PG, Baker HG. 1983. Insects as flower visitors and pollinators. Annual Review of Entomology 28, 407–453.

Kevan PG, Lanet MA. 1995. Flower petal microtexture is a tackle cue for bees. Ecology 76, 4750–4752.

Klein AM, Vaissièere BE, Cane JH, Steffan-Dewenter I, Cunningham SA, Kremen C, Tscharntke T. 2007. Importance of pollinators in changing landscapes for world crops. Proceedings of the Royal Society B: Biological Sciences 274, 303–313.

Luo S-X, Zhang L-J, Yuan S, Ma Z-H, Zhang D-X, Renner SS. 2018. The largest early-diverging angiosperm family is mostly pollinated by ovipositing insects and so are most surviving lineages of early angiosperms. Proceedings of the Royal Society B: Biological Sciences 285, 20172365.

Machado A, Wu Y, Yang Y, Llewellyn DJ, Dennis ES. 2009. The MYB transcription factor GHMYB25 regulates early fibre and trichome development. The Plant Journal 59, 52–62.

Martin C, Bhatt K, Baumann K, Jin H, Zachgo S, Roberts K, Schwarzsommer Z, Glover B, Perez-Rodrigues M. 2002. The mechanics of cell fate determination in petals. Philosophical Transactions of the Royal Society B: Biological Sciences 357, 809–813.

Martin C, Paz-Ares J. 1997. MYB transcription factors in plants. Trends in Genetics 13, 67–73.

Noda K, Glover BJ, Linstead P, Martin C. 1994. Flower colour intensity depends on specialized cell shape controlled by a MYB-related transcription factor. Nature 369, 661–664.

Ojeda I, Francisco-Ortega J, Cronk QCB. 2009. Evolution of petal epidermal morphology in Leguminosae and its use as a marker of petal identity. Annals of Botany 104, 1099–1110.

Ojeda DI, Valido A, Fernandez de Castro AG, Ortega-Olivencia A, Fuertes-Aguilar J, Carvalho JA, Santos-Guerra A. 2016. Pollinator shifts drive petal epidermal evolution on the Macaronesian Islands bird-flowered species. Biology Letters 12, 20160222.

Perez-Rodriguez M, Jaffe FW, Butelli E, Glover BJ, Martin C. 2005. Development of three different cell types is associated with the activity of a specific MYB transcription factor in the ventral petal of Antirrhinum majus flowers. Development 132, 359–370.

Rands SA, Glover BJ, Whitney HM. 2011. Floral epidermal structure and flower orientation: getting to grips with awkward flowers. Arthropod-Plant Interactions 5, 279–285.

Rudall PJ, Remizova MV, Prenger N, Pychid CJ, Tuckett RE, Sokoloff DD. 2009. Nonflowers near the base of extant angiosperms? Spatiotemporal arrangement of organs in reproductive units of Hydatellaceae and its bearing on the origin of the flower. American Journal of Botany 96, 67–82.

Rudall PJ, Sokoloff DD, Remizova MV, Conran JG, Davis JJ, Macfarlane TD, Stevenson DW. 2007. Morphology of Hydatellaceae, an anomalous aquatic family recently recognized as an early-divergent angiosperm lineage. American Journal of Botany 94, 1073–1092.

Schiestl FP, Johnson SD. 2013. Pollinator-mediated evolution of floral signals. Trends in Ecology and Evolution 28, 307–315.

Schneider EL, Jeter JM. 1982. Morphological studies of the Nymphaeaceae. XII. The floral biology of Cabomba caroliniana. American Journal of Botany 69, 1410–1419.

Stracke R, Werber M, Weisshaar B. 2001. The R2R3-MYB gene family in Arabidopsis thaliana. Current Opinion in Plant Biology 4, 447–456.

Taneda H, Watanabe-Taneda A, Chhetry R, Ikeda H. 2015. A theoretical approach to the relationship between wettability and surface microstructures of epidermal cells and structured cuticles of flower petals. Annals of Botany 115, 923–937.

Taylor ML, Williams JH. 2009. Consequences of pollination syndrome evolution for post-pollination biology in an ancient angiosperm family. International Journal of Plant Sciences 170, 584–598.

Thien LB, Bernhardt P, Devall MS, Chen Z-D, Luo Y-B, Fan J-H, Yuan L-C, Williams JH. 2009. Pollination biology of basal angiosperms (ANITA grade). American Journal of Botany 96, 166–182.

Viallette-Guiraud ACM, Alaux M, Legeai F, Finet C, Chambrier P, Brown SC, Chauvet A, Magdalena C, Rudall PJ, Scott CP. 2011. Cabomba as a model for studies of early angiosperm evolution. Annals of Botany 108, 589–598.

Vogel S. 1990. The role of scent glands in pollination. New Delhi: A. M. Reprint Publishing Company.

Warner KA, Rudall PJ, Frohlich MW. 2008. Differentiation of perianth organs in Nymphaeaceae, Taxon 57, 1096–1109.

Warner KA, Rudall PJ, Frohlich MW. 2009. Environmental control of sepalness and petalness in perianth organs of waterlilies: a new Mosaic Theory for the evolutionary origin of a differentiated perianth. Journal of Experimental Botany 60, 3559–3574.

Whitney HM, Chittka L, Bruce TJ A, Glover BJ. 2009. Conical epidermal cells allow bees to grip flowers and increase foraging efficiency. Current Biology 19, 948–953.

Zini LM, Galati BG, Ferrucci MS. 2017. Perianth organs in Nymphaeaceae: comparative study on epidermal and structural characters. Journal of Plant Research 130, 1047–1060.