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Bilirubin present in diverse angiosperms

Cary Pirone[1]*, Jodie V. Johnson[2], J. Martin E. Quirke[3], Horacio A. Priestap[1] and David Lee[1]

[1] Department of Biological Sciences, Florida International University, 11200 SW 8 St., OE-167, Miami, FL 33199, USA
[2] Department of Chemistry, University of Florida, PO Box 117200, Gainesville, FL 3261, USA
[3] Department of Chemistry and Biochemistry, Florida International University, 11200 SW 8 St., CP-304, Miami, FL 33199, USA

Abstract

Background and aims	Bilirubin is an orange-yellow tetrapyrrole produced from the breakdown of heme by mammals and some other vertebrates. Plants, algae and cyanobacteria synthesize molecules similar to bilirubin, including the protein-bound bilins and phytochromobilin which harvest or sense light. Recently, we discovered bilirubin in the arils of *Strelitzia nicolai*, the White Bird of Paradise Tree, which was the first example of this molecule in a higher plant. Subsequently, we identified bilirubin in both the arils and the flowers of *Strelitzia reginae,* the Bird of Paradise Flower. In the arils of both species, bilirubin is present as the primary pigment, and thus functions to produce colour. Previously, no tetrapyrroles were known to generate display colour in plants. We were therefore interested in determining whether bilirubin is broadly distributed in the plant kingdom and whether it contributes to colour in other species.
Methodology	In this paper, we use HPLC/UV and HPLC/UV/electrospray ionization-tandem mass spectrometry (HPLC/UV/ESI-MS/MS) to search for bilirubin in 10 species across diverse angiosperm lineages.
Principal results	Bilirubin was present in eight species from the orders Zingiberales, Arecales and Myrtales, but only contributed to colour in species within the Strelitziaceae.
Conclusions	The wide distribution of bilirubin in angiosperms indicates the need to re-assess some metabolic details of an important and universal biosynthetic pathway in plants, and further explore its evolutionary history and function. Although colour production was limited to the Strelitziaceae in this study, further sampling may indicate otherwise.

Introduction

Tetrapyrroles occur throughout the plant kingdom; this class of molecules includes vital biosynthetic products such as chlorophyll and heme. In plants, the degradation of heme forms first biliverdin-IXα, and subsequently phytochromobilin, the precursor of the phytochrome chromophore, an essential light-sensing molecule (Tanaka and Tanaka 2007). In mammals and some

vertebrates, biliverdin-IXα is also formed from the degradation of heme, but it is transformed into the yellow-orange pigment bilirubin-IXα. We have identified bilirubin-IXα (henceforth referred to as bilirubin) as the major pigment in the orange arils of *Strelitzia nicolai*, the White Bird of Paradise Tree (Pirone et al. 2009). Although ubiquitous in animals, this is the first example of bilirubin in a plant. Subsequently, we have discovered this pigment

* Corresponding author's e-mail address: cpiro001@fiu.edu

in the sepals and arils of *Strelitzia reginae*, the Bird of Paradise Flower, indicating that the pigment is not unique to *S. nicolai* (Pirone *et al.* 2010).

In *S. nicolai* and *S. reginae*, bilirubin is a novel biosynthetic source of display colour. As a rule, the colouration of flowers and fruits is achieved with products from three metabolic pathways: the terpenoid (carotenoids), the phenylpropanoid (flavonoids) and the betalain (betalains) (Davies 2004; Grotewold 2006; Lee 2007). Betalain synthesis is restricted to families in the order Caryophyllales, while carotenoids and flavonoids (including anthocyanins) are pervasive in the plant kingdom (Harborne 1967; Goodwin 1988). A rare group of pigments, the phenalenones, has been documented in several species in the Strelitziaceae and related families (Davies 2004). However, to our knowledge, neither the phenalenones nor the other rare pigments play a significant role in colour production. Bilirubin is thus the first product of an additional biosynthetic route, the tetrapyrrole pathway, to produce conspicuous colour in a plant reproductive structure. Chlorophylls, which are also synthesized via the tetrapyrrole pathway, primarily produce colour in foliage, thus forming a green background upon which the contrasting colours of flowers and fruits are displayed. While chlorophylls occasionally produce colour in reproductive structures, these are fairly inconspicuous.

Given the presence of bilirubin in *Strelitzia*, it is interesting to determine whether the pigment is produced by other taxa within the Strelitziaceae, in families closely allied to the Strelitziaceae (as in the Zingiberales), as well as throughout the major groups of the angiosperms. Preliminary high-performance liquid chromatography (HPLC/UV) analyses of aril extracts of an additional species in the Strelitziaceae, *Phenakospermum guyanense*, showed a pigment with a retention time and UV-Visible spectra that matched those of bilirubin. Here, we use HPLC/UV and HPLC/UV/electrospray ionization-tandem mass spectrometry (HPLC/UV/ESI-MS/MS) to confirm the presence of bilirubin in *P. guyanense* and investigate the presence of bilirubin in the mature fruits from nine additional species and the flowers of a single additional species. Six species are within the order Zingiberales, and four are from diverse angiosperm orders (Table 1). We discuss our findings within a phylogenetic and biochemical context, and comment on a possible ecological role for bilirubin as a colour signal to attract animal dispersers and pollinators.

Materials and methods

Plant material was collected from Fairchild Tropical Botanic Garden in Miami, FL, except aril tissue from *S. reginae*, which was obtained from Ellison Horticulture

Table 1 Summary of HPLC/UV and MS/MS results of the analysis of BR in 10 angiosperm species. N/A indicates that samples were not treated with diazomethane.

Species	Family	Order	Organ	BR detection via diazomethane derivative	BR detection via HPLC/UV	BR detection via HPLC-MS/MS	BR concentration Sample 1	BR concentration Sample 2	Mean BR concentration (n = 2)
M. balbisiana	Musaceae	Zingiberales	Peel	N	N	Y	<44 ng g^{-1}	<44 ng g^{-1}	—
H. collinsiana	Heliconiaceae	Zingiberales	Fruit	N	N	Y	—	<44 ng g^{-1}	—
C. lucanusianus	Costaceae	Zingiberales	Flower	N	N	Y	<44 ng g^{-1}	<44 ng g^{-1}	—
R. madagascariensis	Strelitziaceae	Zingiberales	Aril	N/A	Y	Y	0.001 mg g^{-1}	0.001 mg g^{-1}	0.001 mg g^{-1}
P. guyanense	Strelitziaceae	Zingiberales	Aril	N/A	Y	Y	3.041 mg g^{-1}	5.787 mg g^{-1}	3.725 mg g^{-1}
H. coronarum	Zingiberaceae	Zingiberales	Aril	N	Y	Y	<44 ng g^{-1}	<44 ng g^{-1}	—
G. crispa	Arecaceae	Arecales	Fruit	N	N	Y	<44 ng g^{-1}	<44 ng g^{-1}	—
P. odoratissimus	Pandanaceae	Pandanales	Fruit	N	N	N	—	—	—
P. americana	Lauraceae	Laurales	Fruit	N	N	N	—	—	—
E. luschnathiana	Myrtaceae	Myrtales	Fruit	N	N	Y	<44 ng g^{-1}	—	—

Pty. Ltd in Allstonville, New South Wales, Australia. Tissue for each sample and its replicate were composed of tissue from one or multiple inflorescences or infructescences from a single, sometimes clonal, individual. The replicate aril samples of *P. guyanense* came from different individuals (collected by John Kress; Guyana (South America), Demerara-Mahaica region). For the names and taxonomic affiliations of species sampled, see Table 1. We sampled species from each banana group family, except from the Lowiaceae. This monotypic family consists of 15 rare species within *Orchidantha*, and we were not able to obtain enough material for analysis. We selected *Musa balbisiana* (Musaceae), one of the wild progenitors of most cultivated bananas (Heslop-Harrison and Schwarzacher 2007), *Heliconia collinsiana* (Heliconiaceae) and representatives from each of the two Strelitziaceae genera not previously analysed for bilirubin content, *P. guyanense* and *Ravenala madagascariensis*. We also sampled species from two of the most derived families in the order, *Costus lucanusianus* (Costaceae) and *Hedychium coronarium* (Zingiberaceae) (Kress *et al.* 2001). We mainly sampled orange fruits to maximize the potential chances of finding bilirubin, but we also included the blue arils of *R. madagascariensis*, the yellow fruits of *H. collinsiana* and the multi-coloured flowers of *C. lucanusianus*. To determine whether bilirubin is present in plants outside of the Zingiberales, we sampled species from the basal dicot order Laurales, two monocot orders, the Arecales and the Pandanales, and the eudicot order Myrtales, which is part of the Rosid clade. Selection of species within those orders (Table 1) was based on tissue availability and fruit colour. For each sample (except aril samples), 20.0 g of fresh tissue were ground in a blender with 100 mL of methanol for 2 min, and then filtered through a Buchner funnel. The residue was re-extracted with chloroform in a mortar and pestle. Methanol and chloroform extracts were pooled, and 100 mL of water were added. The mixture was left in a separatory funnel for 5 min, and then the (lower) chloroform layer was collected, filtered with a polytetrafluoroethylene 0.2 μm filter and divided into two equal aliquots. Each aliquot was dried to completion in a rotovap at 30 °C. For each aril sample, 0.05 g of tissue from a single aril was ground by a mortar and pestle and extracted with chloroform repeatedly until the chloroform extracts were colourless. As above, the chloroform extract was filtered, divided into two equal aliquots and dried in a rotovap. All tissues were sampled in duplicate. To determine the presence of bilirubin, one aliquot from each sample was analysed via HPLC and the second aliquot was analysed via HPLC/ESI-MS/MS.

HPLC/UV

HPLC/UV analyses were performed on a Thermo-Finnigan SpectraSystem HPLC apparatus with a variable wavelength photodiode array detector (SMC1000, P4000, AS3000, UV6000LP; Thermo Electro Corporation, San Jose, CA, USA). The extract was re-dissolved in dimethyl sulfoxide (DMSO), partitioned with hexane to remove lipids and chromatographed on a reverse phase ODS-A column (150 mm × 4.3 mm, particle size 5 μm; Waters, Milford, MA, USA). Mobile phase A was 0.1% formic acid in methanol, and mobile phase B was 0.1% formic acid in water. The HPLC gradient (at 1.0 mL min^{-1}) was started at 40% A and increased linearily to 95% A over 40 min, then held constant at 95% A and 5% B for 10 min. Bilirubin was identified by comparing the retention time and UV-Visible spectra of sample pigments with bilirubin standard (Sigma-Aldrich; St Louis, MO, USA), which had a retention time of 42.9 min and a maximum absorbance at 444 nm in the above HPLC solvent system. Bilirubin concentrations were determined by comparison with a standard curve [($R^2 = 0.995$) estimated detection limit = 20 ng injected on column]. Preliminary analysis of some plant extracts showed compounds which eluted at retention times similar to that of bilirubin. The UV-Visible spectra of these compounds were similar to those of carotenoids. To avoid the possible overlap of the HPLC/UV spectra of these pigments with that of bilirubin, we treated non-arillate samples (Table 1) with diazomethane to convert bilirubin to its di-methyl ester, i.e. both carboxylic acids were converted to methyl esters ($\lambda_{max} = 453$ nm in HPLC solvents described above) (Kuenzle *et al.* 1973). Diazomethane was prepared from diazald according to Vogel *et al.* (1989). Chloroform extracts of the sepals were treated with an excess of a solution of diazomethane in order to form the bilirubin di-methyl ester. The addition of the diazomethane was deemed to be complete when effervescence was no longer observed. The excess diazomethane was destroyed by the addition of a few drops of acetic acid. Although the diazomethane would also methylate any other carboxylic acid impurities in the extract, such ester byproducts did not interfere in any way with the observance of the bilirubin di-methyl ester peak in the HPLC analyses. Thus, it was unnecessary to carry out additional purification of the sepal extracts. With the HPLC/UV conditions described above, the retention time of bilirubin di-methyl ester was 31.7 min, thus making it possible to observe the compound without interference from other pigments (Fig. 1). A standard curve for bilirubin di-methyl ester [($R^2 = 1$), estimated detection limit = 35.0 ng injected on column] was constructed by treating bilirubin
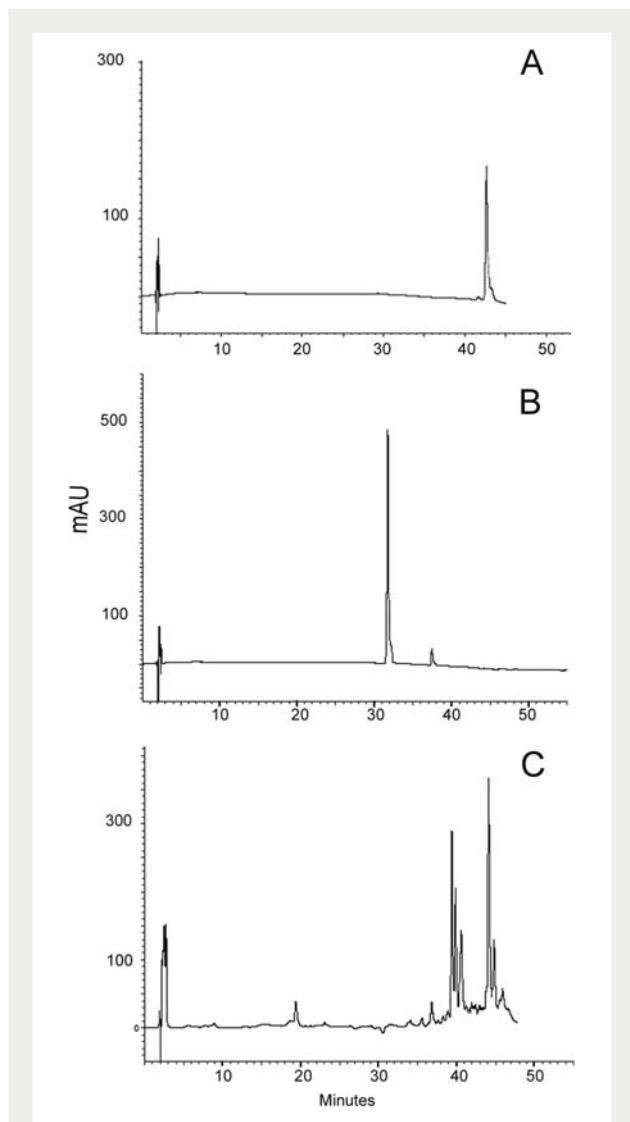
standard with diazomethane. Identification of the peak at 31.7 min as bilirubin di-methyl ester was verified by comparison with bilirubin di-methyl ester standard (Frontier Scientific, Logan, UT, USA), which also eluted at 31.7 min. For samples in which bilirubin was detected via HPLC/ESI-MS/MS but not HPLC/UV, we assumed the mass of bilirubin to be less than the estimated detection limit of bilirubin or bilirubin treated with diazomethane. Standard deviation values were not calculated owing to the low sample size. Instead, concentration values

for the replicate samples of each plant are presented in Table 1.
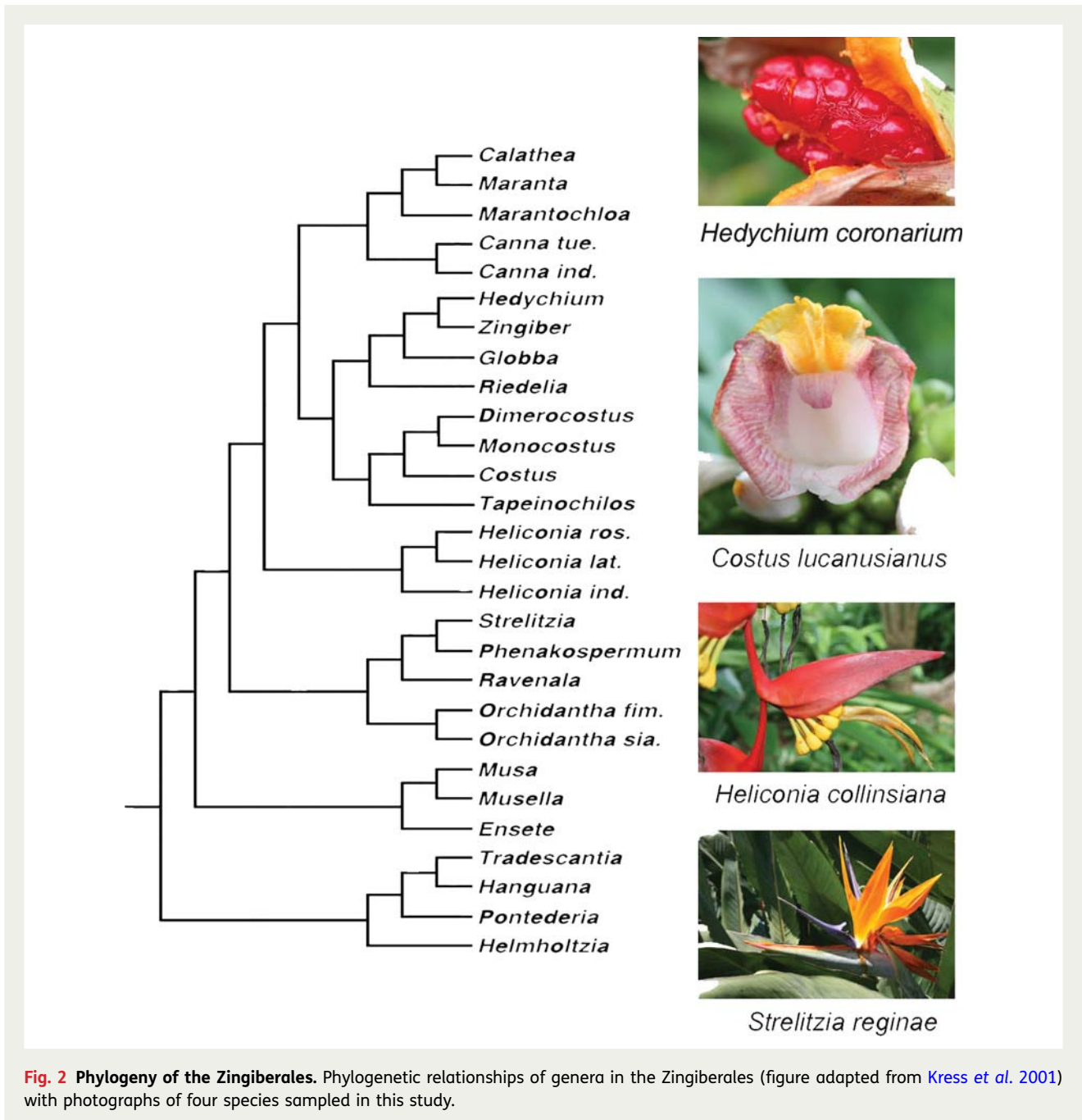
HPLC/UV/ESI-MS/MS

The plant extract was re-dissolved in DMSO (Certified ACS; Fisher Scientific) and analysed via reverse phase C8 HPLC/UV/ESI-MS/MS utilizing both positive and negative electrospray ionization source (ESI) and a number of different MS^n scans. HPLC/UV was performed with an Agilent Technologies HPLC with binary pumps (1100 series; Santa Clara, CA, USA), a Symmetry C8 HPLC column (150 mm × 2.1 mm, particle size 5 μm; Waters) and an Agilent UV-Visible detector (G1314A). The mobile phase A was 0.2% acetic acid (glacial, biochemical grade (99.8%); ACROS organics, Morris Plains, NJ, USA) in H_2O (HPLC grade, Honeywell Burdick & Jackson, Muskegon, MI, USA) and mobile phase B was 0.2% acetic acid in acetonitrile (LC-MS grade, Honeywell Burdick & Jackson). The HPLC gradient (at 0.2 mL min^{-1}) was started at 20% B at time 0 and increased linearly to 85% B over 30 min and then increased linearly to 100% B over 15 min. The column was held at 100% B until monitoring of the UV/MS signal showed no further elution of peaks. For some extracts, this was more than 150 min. The UV-Visible response was monitored at 450 nm for bilirubin, which eluted at ~39 min.

All mass spectrometry data were obtained with a Finnigan MAT (San Jose, CA, USA) LCQ classic quadrupole ion trap mass spectrometer equipped with an ESI. The ESI was operated with a nitrogen sheath and auxiliary gas flows of 65 and 5, respectively (unitless instrument parameters), with a spray voltage of 3.3 kV and a heated capillary temperature of 250 °C. The heated capillary voltage was +15 and −22 V for (+) and (−)ESI, respectively, while the tube lens was operated at 0 V for both ESI polarities. Collision-induced dissociation (CID) was conducted with a parent ion isolation of 3u, CID energy of 37.5%, qCID of 0.25 and CID time of 30 ms.

With (+)ESI, bilirubin produced an *m/z* 585 [M + H]$^+$ ion and an *m/z* 583 ion due to oxidation during ionization. The *m/z* 585 and 583 ions underwent CID-MS/MS to form *m/z* 299 and 297 ions, respectively, as major product ions. With (−)ESI-MS, bilirubin produced *m/z* 583 [M-H]$^-$ and *m/z* 581 ions which were dissociated to form *m/z* 285 and 537 major product ions, respectively. Bilirubin was identified in the plant extracts by matching of retention time and (+) and (−)ESI-MS and −MS/MS spectra with those of the authentic bilirubin standard.

False positives due to contamination during handling and analyses were avoided by washing the glassware multiple times (soap and water, acetone, chloroform), the use of disposable vials when possible and changing gloves after the preparation of each sample. Dimethyl sulfoxide solvent blanks were analysed before and

Fig. 2 Phylogeny of the Zingiberales. Phylogenetic relationships of genera in the Zingiberales (figure adapted from Kress *et al.* 2001) with photographs of four species sampled in this study.

after analyses of each extract and after analyses of bilirubin standards to check for carryover of bilirubin. The DMSO blanks were repeated if significant levels of bilirubin were carried over to subsequent runs.

Results

Bilirubin was present in 8 of the 10 species tested: *M. balbisiana, H. collinsiana, C. lucanusianus,*

R. madagascariensis, P. guyanense, H. coronarium, Gastrococos crispa and *Eugenia luschnathiana* (Table 1). Bilirubin was present in the fruits (including aril and peel) of all species except *C. lucanusianus,* where it was present in the flowers (fruits were unavailable for sampling). In *R. madagascariensis* and *P. guyanense,* HPLC/UV analysis of the aril extracts showed a single peak with a retention time and UV-Visible spectrum that matched those of the bilirubin standard. Bilirubin identification was confirmed

by HPLC/ESI-MS/MS. Bilirubin was also identified in *M. balbisiana*, *H. collinsiana*, *C. lucanusianus*, *H. coronarium*, *G. crispa* and *E. luschnathiana* via HPLC/ESI-MS/MS, but was not detected via HPLC/UV, even after treatment with diazomethane. In *H. collinsiana* and *E. luschnathiana*, bilirubin was detected in only one of the two replicate samples, while in all other species bilirubin was detected in both replicates. The concentration of bilirubin was highly variable among species. Concentrations ranged from <44 ng g^{-1} of fresh tissue ($n = 2$) to 3.73 mg g^{-1} of fresh tissue ($n = 2$) (Table 1).

Discussion

We initially discovered bilirubin in *S. nicolai* (family: Strelitziaceae, order: Zingiberales), and also in the arils and sepals of *S. reginae*. Since similar secondary metabolites are expected to be found within members of a clade, we concentrated most of our sampling within the Strelitziaceae and the Zingiberales. Kress *et al.* (2001) divide the order into two major clades: the basal 'banana group', which includes the Musaceae, Strelitziaceae, Heliconiaceae and Lowiaceae, and the more derived 'ginger group', which includes the four remaining families (Fig. 2). Although the resolution of intrafamilial relationships varies among other studies, the ginger clade families are generally well resolved (Rudall *et al.* 1999; Chase *et al.* 2000; Givnish *et al.* 2006), and there is high support for the position of the Lowiaceae as sister to the Strelitziaceae (Rudall *et al.* 1999; Chase *et al.* 2000; Soltis *et al.* 2000, 2007; Givnish *et al.* 2006).

The detection of bilirubin in *Gastrococos crispa* (family: Arecaceae, order: Arecales) and *E. luschnathiana* (family: Myrtaceae, order: Myrtales) indicates that bilirubin is not restricted to the Zingiberales and may be broadly distributed throughout the plant kingdom. However, the lack of detection of bilirubin in avocado, *Persea americana* (family: Lauraceae, order: Laurales) and *Pandanus odoratissimus* (family: Pandanaceae, order: Pandanales), suggests that bilirubin is not universal in plants at levels detectable by mass spectrometry.

The high concentration of bilirubin in the arils of *P. guyanense* indicates its role in colour production. Previous studies indicated bilirubin is also responsible for colour production in two other Strelitziaceae species, *S. nicolai* and *S. reginae*. Since brightly coloured fruit displays often serve as signals to attract dispersers (Van der Pijl 1982), it is likely that in these species, bilirubin contributes to the attraction of avian frugivores which feed upon the arils (Frost 1980; W. J. Kress, Smithsonian Institute, pers. comm.). Whether bilirubin plays an additional role beyond colour production in plants remains to be determined. Bilirubin may function as a potent

Fig. 3 **Bilirubin production in *S. nicolai* during aril development.** Maturing arils of *S. nicolai* with increasing amounts of bilirubin. Seeds ∼4 mm in diameter.

antioxidant in plants as it does in humans (Stocker *et al.* 1987), or serve a different physiological function. Bilirubin may also be a mere metabolic waste product.

The detection of bilirubin in only one replicate of *H. collinsiana* and *E. luschnathiana*, and the variability of bilirubin concentration within *P. guyanense*, may indicate that bilirubin biosynthesis is not constant, but is instead variable and influenced by factors which are currently unknown. For example, we observed the accumulation of bilirubin in aril cells during the development of *S. nicolai* (Pirone, personal observation), with a maximum quantity present in mature tissue (Fig. 3), suggesting that bilirubin production may be influenced by development. The lack of bilirubin at the time of sampling may also be a function of variable production, and thus may not indicate an absence of bilirubin biosynthesis in the species.

The biochemical pathway that produces bilirubin in plants remains unknown. In animals, biliverdin-IXα is reduced by an NAD(P)H-dependent biliverdin-IXα reductase (BR) to form bilirubin-IXα (Maines and Trakshel 1993). Conversely, in plants, biliverdin-IXα is converted to a structural isomer of bilirubin, 3Z-phytochromobilin, the precursor of the phytochrome chromophore (Tanaka and Tanaka 2007), by a ferredoxin-dependent bilin reductase, phytochromobilin synthase (Terry *et al.* 1995; Kohchi *et al.* 2001). In cyanobacteria and some algae, biliverdin-IXα is also reduced by ferredoxin-dependent enzymes, the bilin reductases, to form the phycobilin chromophores (Beale 1993; Frankenberg *et al.* 2001). A BR enzyme was identified and cloned in the cyanobacterium *Synechocystis* (Schluchter and Glazer 1997), and BR enzymes have also been found in a variety of other bacteria. Whether bilirubin-IXα in plants is formed by the reduction of biliverdin-IXα by a BR enzyme or via some other means remains to be determined.

Conclusions and forward look

Bilirubin was present in eight species from three diverse angiosperm orders, and contributed to aril colour in

species within the Strelitziaceae. Further sampling of bilirubin in plants, both across species, under variable conditions and across different time scales, will be necessary to gain a comprehensive understanding of the distribution of bilirubin in plants, and to determine whether colour production is limited to the Strelitziaceae. These studies, combined with work on the biosynthesis of bilirubin in plants, will provide a more comprehensive understanding of the evolution of this unusual molecule in the plant kingdom.

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Contributions by the authors

C.L.P. wrote the manuscript, collected plants, prepared extracts and performed HPLC analyses. J.V.J. performed and interpreted MS/MS analyses. J.M.Q. also interpreted MS/MS spectra and oversaw lab work. H.P. prepared the *Hedychium* samples and provided general lab advice. D.W.L. was the first to observe the uniqueness of Strelitziaceae pigments, and contributed to the discussion on ecology and colour production.

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Conflict of interest statement

None declared.

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