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Research

Coordination of anthocyanin decline and photosynthetic maturation in juvenile leaves of three deciduous tree species

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Summary

• Juvenile leaves in high-light environments commonly appear red as a result of anthocyanin pigments, which play a photoprotective role during light-sensitive ontogenetic stages. The loss of anthocyanin during leaf development presumably corresponds to a decreased need for photoprotection, as photosynthetic maturation allows leaves to utilize higher light intensities. However, the relationship between photosynthetic development and anthocyanin decline has yet to be quantitatively described.

• In this study, anthocyanin concentration was measured against photopigment content, lamina thickness, anatomical development, and photosynthetic CO_2 exchange in developing leaves of three deciduous tree species.

• In all species, anthocyanin disappearance corresponded with development of *c.* 50% mature photopigment concentrations, *c.* 80% lamina thickness, and differentiation of the mesophyll into palisade and spongy layers. Photosynthetic gas exchange correlated positively with leaf thickness and chlorophyll content, and negatively with anthocyanin concentration. Species with more rapid photosynthetic maturation lost anthocyanin earliest in development. Chlorophyll *a/b* ratios increased with leaf age, and were lower than those of acyanic species, consistent with a shading effect of anthocyanin.

• These results suggest that anthocyanin reassimilation is linked closely with chloroplast and whole-leaf developmental processes, supporting the idea that anthocyanins protect tissues until light processing and carbon fixation have matured to balance energy capture with utilization.

Key words: *Acer rubrum* (red maple), anthocyanin, *Cercis canadensis* (redbud), development, juvenile leaves, *Liquidambar styraciflua* (sweetgum), photoprotection, photosynthesis.

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Introduction

One of the most conspicuous developmental changes observed in juvenile leaves as they mature is color change, with young leaves on new growth tips of many species first appearing red, purple, pink, or, less commonly, blue or white, and becoming greener with leaf age. The red-to-blue coloration of young leaves is most commonly caused by the pigment anthocyanin, appearing within vacuoles of epidermal and/or mesophyll cells within hours to days of seedling germination, and then decreasing concomitantly with leaf expansion and maturation (Choinski *et al.*, 2003; Cai *et al.*, 2005). Anthocyanic coloration of juvenile leaves is nearly ubiquitous in tree species at equatorial latitudes (for species surveys, see Lee & Collins, 2001 and Dominy *et al.*, 2002), where coloration has been observed to precede and accompany chlorophyll development in species

with delayed greening (Kursar & Coley, 1992; Cai *et al.*, 2005). Expanding leaves of both woody perennials (for species survey, see Price & Sturgess, 1938) and herbaceous species of the temperate zone (see studies listed in Chalker-Scott, 1999) also commonly exhibit anthocyanin, but during a more gradual greening process.

Despite its widespread phylogenetic distribution, the functional significance of anthocyanin in juvenile leaves remains unresolved. Some hypotheses, such as the elevation of leaf temperature (Smith, 1909), have been clearly dismissed (Lee *et al.*, 1979, 1987; Hughes, 2004), while others, including camouflage against herbivory (Stone, 1979), fungicide (Coley & Aide, 1989) or antioxidant activity (Rice-Evans *et al.*, 1997; Wang *et al.*, 1997), or a signal indicating the presence of unpalatable phenolics (Hamilton & Brown, 2001) have received mixed support (Coley, 1981, 1983; Kursar & Coley, 1992; Dominy *et al.*, 2002; Neill *et al.*, 2002; Numata *et al.*, 2004; Karageorgou & Manetas, 2006; Manetas, 2006). Perhaps the most compelling current explanation for anthocyanin in developing tissues is that the pigments act as light attenuators, protecting underlying cells from high irradiance through absorption of high-energy blue-green (and possibly UV) wavelengths of the solar spectrum (for reviews, see Chalker-Scott, 1999 and Gould, 2004). While this latter function may not be beneficial to species in deeply shaded habitats, light attenuation should be particularly advantageous to species with leaves that initiate maturation at more sun-exposed, apical tips.

Immature leaves tend to be especially vulnerable to high-light stress because of a combination of several factors, including immature chloroplast structure (Pettigrew & Vaughn, 1998; Choinski *et al.*, 2003), reduced capacity and quantity of photosynthetic enzymes (Miranda *et al.*, 1981a; Pettigrew & Vaughn, 1998), and limited stomatal and cellular conductance of CO_2 (Miranda *et al.*, 1981b; Choinski *et al.*, 2003). As a result, young leaves growing under high irradiances tend to photosynthetically saturate, as well as photoinhibit, under substantially lower sunlight intensities than mature leaves (Hoflacher & Bauer, 1982). It is therefore generally beneficial for light capture to be down-regulated early in leaf development. Observed strategies to decrease light capture in juvenile leaves include increased xanthophyll pigments (Krause *et al.*, 1995; Barker *et al.*, 1997), inclined leaf orientation (Jiang *et al.*, 2006), pubescence (Liakopoulos *et al.*, 2006), decreased chlorophyll content (Choinski *et al.*, 2003; Cai *et al.*, 2005), and light-attenuating layers such as anthocyanin (Manetas *et al.*, 2002; Karageorgou & Manetas, 2006; Liakopoulos *et al.*, 2006). Although the physiological benefits of these processes have been addressed and supported in several studies, little information exists which quantitatively describes the coordination between their down-regulation and the maturation of the photosynthetic processes they are assumed to protect. The objective of the present study was to quantitatively describe the relationship between anthocyanin diminution and photosynthetic maturation, including development of leaf structure, photopigment accumulation, and photosynthetic CO_2 exchange, in three deciduous tree species common in the temperate zone.

Materials and Methods

Pigment composition, lamina thickness and anatomy, and photosynthetic CO_2 exchange were measured for developing leaves of three deciduous tree species under natural field conditions. These parameters were related to the disappearance of visible anthocyanin in order to identify possible functional interactions. Measurements were made at consecutive apical nodes, representing increasing stages of maturation.

Plant material

Species observed in this study were sweetgum (*Liquidambar styraciflua* L., Hamamelidaceae), red maple (*Acer rubrum* L., Aceraceae), and Eastern redbud (*Cercis canadensis* L., Fabaceae) growing along disturbed high sunlight-exposed roadsides in Winston-Salem, NC, USA (36 8′N 80 13′W). For acyanic juvenile leaf comparison of chl*a*/*b* ratios, juvenile leaves of English ivy (*Hedera helix* L., Araliaceae), forsythia (*Forsythia suspensa* Thunb., Oleaceae), and Japanese honeysuckle (*Lonicera japonica* Thunb., Caprifoliaceae) growing in the same areas were also included. All of these species continuously produce new growth throughout the summer, and measurements were taken between July and early September. Since some variation exists in the number of apical red leaves per branch of a given species, we used branches exhibiting the most commonly observed number of red leaves per branch in each study: sweetgum branches most commonly had two apical red leaves, while red maple had three, and redbud had four (Fig. 1). Leaves were determined to be without anthocyanin when anthocyanin concentrations were less than 60 μmol m^{-2}, corresponding to values where anthocyanins were no longer visible to the naked eye. In all experiments, at least five different trees growing in fully exposed (i.e. > 7 h d^{-1} full sunlight) habitats were sampled, using one branch on the south side of each tree. All trees were saplings < 3 m in height. The most basal leaves of the same branch on which juvenile leaves were sampled were used to represent 'mature' leaves. Maturity was assumed only if leaf thickness, photosynthesis, and chlorophyll contents remained constant compared with younger leaves on the branch. All mature leaves were also exposed to full irradiance (> 1350 μmol m^{-2} s^{-1}) during much of the day, including the time of sampling.

Microscopic sectioning

Branches of each species were removed from plants, re-cut underwater, and kept hydrated until sectioned into 50–100 μm sections using a vibratome. Sections were mounted on a Zeiss Axioplan upright microscope (Carl Zeiss Inc., Thornwood,

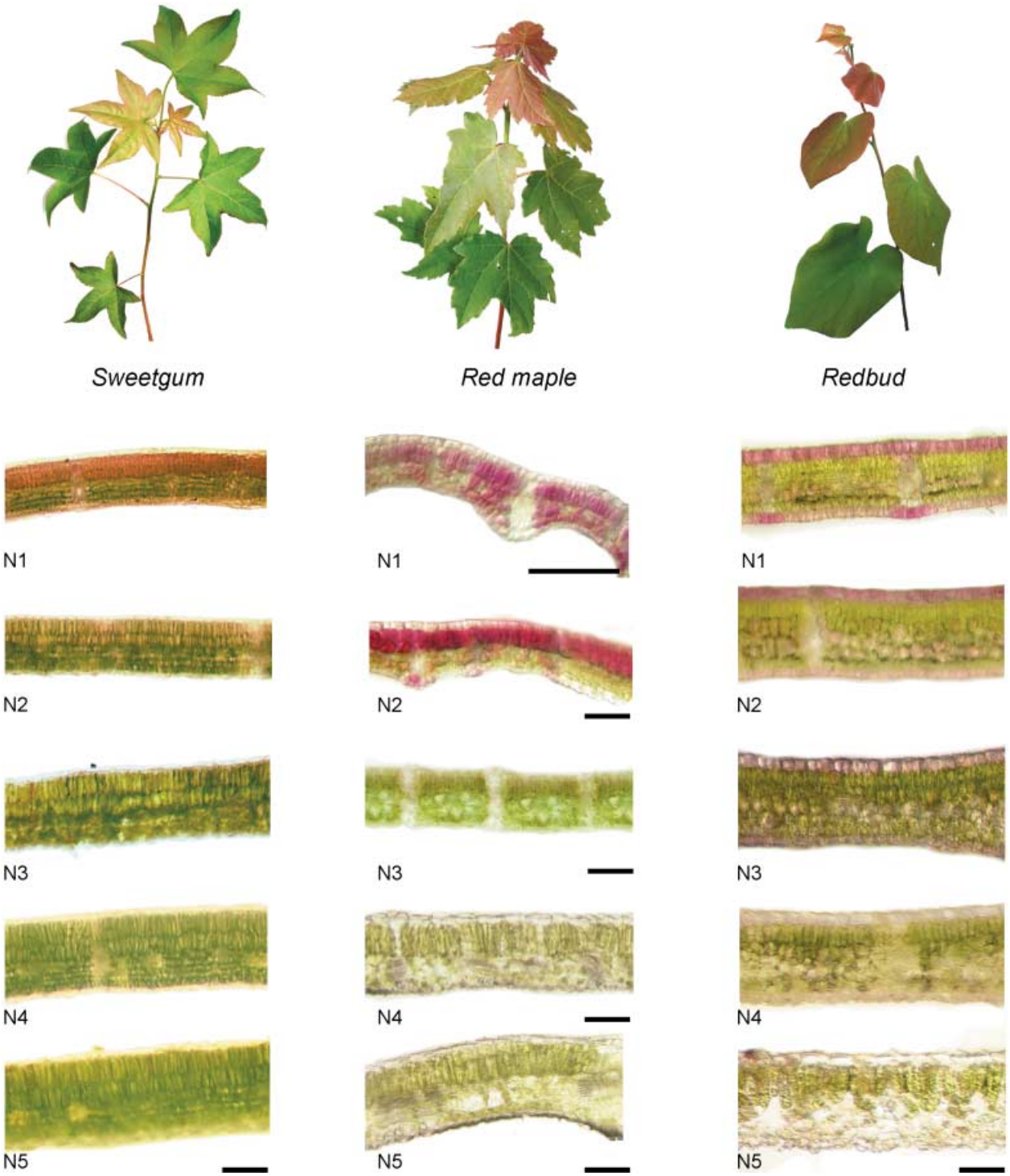

Fig. 1 Whole branches and microscopic sections of leaves from nodes 1–5 (N1–N5) of *Liquidambar styraciflua* (sweetgum), *Acer rubrum* (red maple), and *Cercis canadensis* (redbud). Bars, 100 μm. In sweetgum, leaf sections were magnified at ×100; red maple, ×200 for node 1, ×100 for nodes 4–5; redbud, ×200.

NY, USA), viewed using differential interference contrast (DIC) microscopy, and images were captured using a Hamamatsu C5810 three-chip cooled color CCD camera (Hamamatsu Photonics, Hamamatsu City, Japan). Photographs of leaf sections were rotated and adjusted for brightness and image sharpness using Adobe Photoshop CS (Adobe Systems, San Jose, CA, USA). The backgrounds of all images were also converted to white using Photoshop.

Leaf thickness

Six whole branches of each species were cut from trees between 10:00 and 11:00 h, re-cut underwater, and remained submerged until analysis within 2 h. During this time, no visible wilting was observed to occur, and leaf thicknesses were not found to differ significantly from nonexcised branches (data not shown). Three to six pieces (depending on variance) of leaf tissue visibly free of protruding veins were cut out from along the midrib of each leaf and immediately measured using IP54 electronic micrometer calipers with 1 μm precision (Fred V. Fowler Co., Inc, Newton, MA, USA). Average values were compared with those of microscopic sections measured using a reticle to ensure accuracy. No significant difference was observed, as microscopic values fell within ranges derived using calipers (data not shown). Mean thickness of each leaf was divided by the average thickness of fully expanded leaves of that species to estimate percentage of mature leaf thickness for each node.

Pigment quantification

Branches were collected between 10:00 and 11:00 h, during which time all plants had been exposed to full sunlight for at least 3 h. A standard hole puncher was used to excise three 0.33 cm^2 leaf discs, which were then placed in 3 ml *N,N′*-dimethylformamide to extract in the dark for 24 h. Chlorophyll concentrations were determined spectrophotometrically using the equations described by Porra (2002), and total carotenoid concentrations were calculated using equations described by Wellburn (1994). Chlorophyll concentrations were reported as a function of both volume (using mean leaf thicknesses calculated for each node, derived from other branches previously measured) and leaf area. This was done to determine whether increases in chlorophyll content corresponded to increased leaf thickness, as opposed to increased intracellular chlorophyll (the former being evidenced by changes in chlorophyll per unit area, but not volume). From the same leaves, three additional leaf discs were excised and placed in 3 ml 6 M HCl : H_2O : MeOH (7 : 23 : 70) for 24 h at 4 C to extract anthocyanins. Anthocyanin concentration was measured spectrophotometrically as $A_{530} - 0.24A_{653}$ using an extinction coefficient of 30 000 L mol^{-1} cm^{-1} (Murray & Hackett, 1991). All extracts were analyzed using a Hewlett Packard 8453 UV-VIS spectrophotometer (Hewlett Packard, Palo Alto, CA, USA). Concentrations of chlorophyll and carotenoid pigments for leaves of each node were divided by average values of fully expanded leaves to estimate the percentage of leaf pigments present relative to mature leaves.

Chlorophyll *a/b* ratios were calculated to assess relative emphasis on light capture vs processing during maturation, as higher chlorophyll *a/b* ratios reflect an increase in core (exclusively chl*a*) relative to light-harvesting (both chl*a* and *b*) complexes, and/or higher ratios of photosystem I (PSI, 4/1 ratio of chl*a/b*) to photosystem II (PSII, 1.2/1 ratio) (Cui *et al.*, 1991; Demmig-Adams, 1998; Hopkins & Hüner, 2004a). Carotenoid/chlorophyll (car/chl) ratios were also calculated to compare relative photoprotection by the xanthophylls through leaf development to photosynthetic maturity.

Photosynthetic gas exchange

A portable photosynthesis system (PP Systems Inc. model TPS-1, Amesbury, MA, USA) was used to measure leaf conductance and photosynthesis for plants in the field. Measurements were taken between 09:00 and 12:00 h on plants under clear-sky ambient sunlight (> 1350 μmol m^{-2} s^{-1}) after the leaf had been enclosed in the cuvette for at least 1 min and oriented directly towards the sun, to allow for light-saturated photosynthesis (leaf temperatures rarely exceeded ambient by > 1 C). Measured light response curves of all age-class leaves were light-saturated (> 85% of mean maximum values) at well below (< 48%) full sunlight (data not shown), consistent with the findings of Hoflacher & Bauer (1982) and Wallace & Dunn (1980). Thus, measured photosynthesis under full sunlight was assumed to represent light-saturated photosynthesis (A_{sat}) under ambient CO_2. The most basal (nonsenescent) leaves on the branch were measured to represent fully mature leaves. Leaf area was measured using a Delta-T leaf area measurement system (Delta-T Devices Ltd, Cambridge, UK) and photosynthesis was expressed on a per-unit-leaf-area (projected) and -volume basis. Leaf mass was not used to express photosynthesis because of potential problems associated with variations in mass per unit volume (e.g. cell wall development).

Statistics

To compare concentrations of photosynthetic pigments, leaf thickness, and gas exchange of youngest nodes lacking visible anthocyanin, measurements for the first nonred leaf node (node 3 for sweetgum, node 4 for red maple, and node 5 for redbud) were compared using a single-factor ANOVA with Tukey–Kramer test for means difference. ANOVA with Tukey–Kramer test was also used to compare these parameters for fully mature leaves. A Pearson product moment correlation test was used to examine the relationship between leaf node and pigment concentrations, pigment ratios, leaf thicknesses, and photosynthesis for individual leaves (as opposed to means). Sample size (N) was five or greater for all comparisons, with N – 1 degrees of freedom. Significance for all tests was determined at $P < 0.05$.

Results

Leaf anatomy

Anthocyanins were contained within either the epidermis (redbud) or mesophyll cells (red maple and sweetgum) of

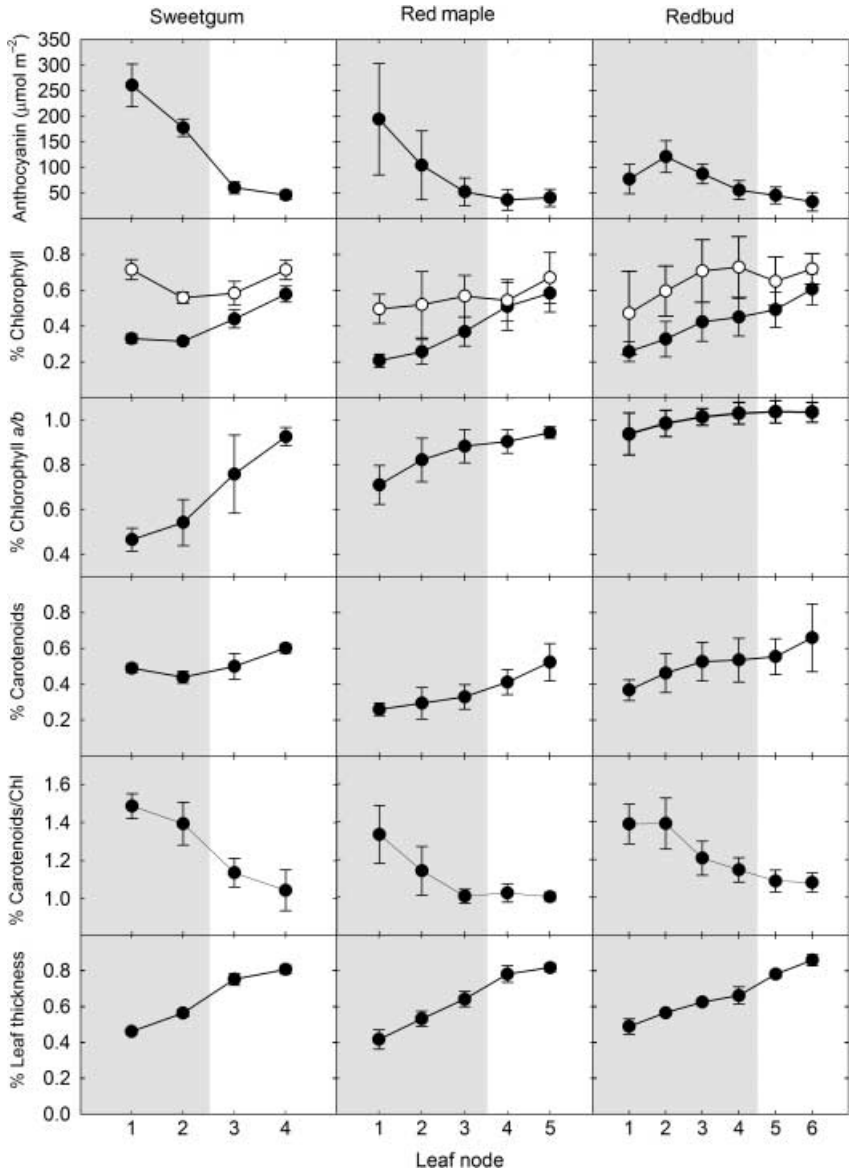

Fig. 2 Pigment concentrations and leaf thicknesses expressed as a percentage of mature values. Shaded areas represent visible presence of anthocyanin. Illustrated are: anthocyanin content, percentage chlorophyll content per unit area (closed circles) and per unit volume (open circles), percentage chlorophyll *a*/*b*, percentage total carotenoids (per unit area), and percentage leaf thickness for *Liquidambar styraciflua* (sweetgum), *Acer rubrum* (red maple) and *Cercis canadensis* (redbud). Points represent means of five replicates ± SD for pigments, and ± SE for lamina thickness.

developing leaves (Fig. 1). Red maple and sweetgum contained significantly more anthocyanin per unit area than redbud in the youngest two leaf nodes ($P < 0.0001$) (Fig. 2), likely reflecting differences in mesophyll vs epidermal pigmentation. In red maple and sweetgum, all mesophyll cells initially exhibited reddening, although anthocyanins eventually became restricted to palisade cells in both species before their disappearance. While reddening was observed to occur in upper (adaxial) cells of all species, the youngest leaves of redbud often exhibited intense reddening in abaxial epidermal cells, which dissipated as leaves matured. Anthocyanins were also observed in abaxial epidermal cells surrounding protruding leaf veins in redbud and red maple, even when other abaxial epidermal and/or mesophyll cells contained little or no visible anthocyanin. In all species, anthocyanins remained visible until palisade cells were visibly differentiated, and had elongated to comprise > 40% of the mesophyll (Fig. 1).

Sweetgum leaves were significantly thicker than the other species during early development ($P = 0.022$ for node 1, $P = 0.0036$ for node 2, $P < 0.0001$ for node 3), but only significantly thicker than redbud in later development through maturity ($P < 0.0001$ for node 4, $P < 0.0001$ for mature leaves) (Table 1). Thickness of red maple leaves did not significantly differ from redbud leaves until node 4, after which they became increasingly thicker ($P < 0.0001$ for node 4, $P < 0.0001$ for node 5, $P = 0.0002$ for mature leaves). At the time of anthocyanin disappearance, lamina thickness relative to mature leaves upon anthocyanin loss did not differ significantly

Table 1 Mean developmental characteristics (with standard deviation) for each species by leaf node

	Node	Chl*a* (mg m^{-2})	Chl*b* (mg m^{-2})	Total chl (mg m^{-2})	Total chl (kg m^{-3})	Chl*a*/*b* (g g^{-1})	Total carotenoids (mg m^{-2})	Carotenoid/ chl (g g^{-1})	Leaf thickness (μm)	A_{sat} (μmol m^{-2} s^{-1})	A_{sat} (mmol m^{-3} s^{-1})	*g* (mmol m^{-2} s^{-1})
Liquidambar styraciflua	1	62.3 (7.2)	41.7 (2.1)	104 (8.0)	0.976 (0.076)	1.49 (0.16)	59.4 (3.0)	0.572 (0.025)	106 (5.3)	0.310 (1.7)	2.91 (16)	97.4 (35)
	2	62.7 (6.2)	36.6 (4.3)	99.4 (5.6)	0.762 (0.043)	1.74 (0.33)	53.2 (4.1)	0.537 (0.043)	130 (11)	8.76 (2.0)	67.2 (16)	140 (37)
	3	96.5 (9.8)	41.8 (12)	138 (16)	0.797 (0.091)	2.43 (0.56)	60.6 (8.9)	0.437 (0.029)	174 (17)	11.5 (2.4)	66.4 (14)	143 (24)
	4	136 (10)	46.0 (4.1)	182 (14)	0.975 (0.076)	2.96 (0.13)	72.7 (3.1)	0.402 (0.041)	186 (13)	13.3 (2.3)	71.1 (13)	137 (30)
	Mature	240 (20)	75.2 (8.1)	315 (28)	1.36 (0.12)	3.20 (0.18)	121 (9.0)	0.385 (0.0069)	231 (11)	11.6 (2.6)	50.7 (11)	171 (90)
Acer rubrum	1	74.1 (17)	30.6 (3.9)	105 (10)	1.11 (0.18)	2.40 (0.30)	50.8 (9.4)	0.477 (0.055)	85.5 (19)	–0.156 (1.1)	–1.56 (12)	48.1 (18)
	2	90.1 (17)	31.5 (5.1)	122 (22)	1.16 (0.41)	2.85 (0.13)	49.2 (7.1)	0.408 (0.046)	109 (21)	2.45 (0.73)	22.5 (6.7)	66.6 (27)
	3	150 (26)	49.6 (8.9)	200 (35)	1.27 (0.26)	3.04 (0.067)	72.2 (14)	0.360 (0.013)	132 (22)	3.75 (1.7)	28.4 (13)	84.2 (53)
	4	228 (23)	70.0 (7.4)	298 (30)	1.22 (0.26)	3.25 (0.073)	109 (7.6)	0.366 (0.017)	160 (23)	6.11 (1.9)	38.1 (12)	104 (41)
	5	241 (18)	72.1 (4.8)	313 (22.7)	1.50 (0.31)	3.34 (0.074)	112 (8.7)	0.360 (0.0081)	168 (11)	8.05 (1.37)	48.0 (8.2)	120 (28)
	Mature	375 (95)	108 (30)	483 (120)	2.35 (0.061)	3.49 (0.14)	172 (40)	0.358 (0.012)	205 (29)	9.58 (2.7)	46.7 (13)	200 (110)
Cercis canadensis	1	64.7 (15)	18.9 (3.1)	83.6 (18)	1.06 (0.23)	3.39 (0.34)	38.8 (6.0)	0.470 (0.036)	78 (15)	–0.672 (1.3)	5.07 (9.5)	49.4 (43)
	2	83.0 (25)	23.2 (6.9)	106 (32)	1.16 (0.35)	3.56 (0.21)	48.9 (11)	0.470 (0.046)	91.2 (2.3)	0.442 (0.88)	4.85 (9.7)	44.85 (20)
	3	108 (28)	29.5 (7.6)	137 (35)	1.37 (0.35)	3.67 (0.13)	55.5 (11)	0.408 (0.030)	101 (2.1)	2.49 (1.4)	24.7 (14)	76.9 (22)
	4	115 (26)	31.1 (7.9)	146 (34)	0.88 (0.20)	3.73 (0.17)	56.3 (13)	0.387 (0.022)	107 (17)	5.3 (1.2)	49.7 (11)	129 (31)
	5	126 (24)	33.6 (7.6)	159 (32)	1.27 (0.25)	3.75 (0.18)	58.3 (10)	0.367 (0.020)	126 (7)	6.98 (1.5)	55.5 (12)	130 (68)
	Mature	254 (29)	70.1 (7.4)	324 (36)	2.01 (0.22)	3.62 (0.16)	110 (14)	0.338 (0.0092)	161 (11)	11.7 (1.4)	72.4 (8.5)	239 (35)

A_{sat}, light-saturated photosynthesis; chl, chlorophyll; *g*, leaf conductance.
Dashed lines represent the transition between visible (above line) and nonvisible (below line) anthocyanin. All values except chlorophyll expressed per unit volume significantly increase (or decrease in the case of carotenoid/chlorophyll ratios) with node number ($P < 0.01$).

($P = 0.596$) between species, and complete disappearance had occurred by the time leaves were *c.* 80% of mature leaf thickness (Fig. 2).

Pigment accumulation

In all three species, leaf chlorophyll concentration significantly increased with node position when expressed on a leaf area basis ($r^2 = 0.77$, 0.68, and 0.48 for sweetgum, red maple, and redbud, respectively; $P < 0.0001$ in all cases; Table 1, Fig. 2). By contrast, chlorophyll concentrations did not significantly correlate with leaf node when expressed on a leaf volume basis ($r^2 = 0.00$, 0.15, 0.14 and $P = 0.91$, 0.060, 0.061 for sweetgum, red maple, and redbud, respectively) (Table 1, Fig. 2). All three species had microscopically visible anthocyanin until mean values of 44% of total chlorophyll per unit area (relative to mature leaves) had developed. This percentage did not vary significantly between species ($P = 0.262$). For chlorophyll expressed on a leaf volume basis, the percentage was 58% and also did not differ among species ($P = 0.182$). Anthocyanins were retained until *c.* 49% of mature-leaf carotenoids (per unit area) had developed in all three species ($P = 0.058$).

When absolute pigment concentrations were compared (as illustrated in Table 1), as opposed to percentages of maturation,

anthocyanin in sweetgum leaves disappeared significantly earlier ($P < 0.01$) with regard to chlorophyll content on a unit-volume basis compared with red maple and redbud (which did not differ). This translated into 0.797 kg m^{-3} for sweetgum, and 1.22 and 1.27 kg m^{-3} for red maple and redbud, respectively. On a unit area basis, anthocyanin loss also occurred significantly earlier in sweetgum compared with red maple ($P = 0.048$), but did not significantly differ from redud. On average, sweetgum leaves lost anthocyanin by the time 138 mg m^{-2} chlorophyll had developed, while red maple and red bud retained anthocyanin until 298 and 159 mg m^{-2} chlorophyll had developed, respectively. Carotenoid concentrations did not significantly vary between species in this respect ($P = 0.19$), as anthocyanins had disappeared by the time mean carotenoid concentrations reached 63 mg m^{-2} in all species.

While leaf chlorophyll *a/b* ratios decreased from the tip down in acyanic species (honeysuckle, $r^2 = 0.416$; ivy, $r^2 = 0.330$; forsythia, $r^2 = 0.561$; $P < 0.005$ for all species), chl*a/b* significantly increased in all three anthocyanic species ($r^2 = 0.76$, 0.56, and 0.31 for sweetgum, red maple, and redbud, respectively; $P < 0.01$ for all species) (Fig. 2, Table 1). The range of chl*a/b* values varied substantially among the anthocyanic species. Sweetgum leaves had the broadest range and lowest chl*a/b* ratios of all species, ranging between 1.5 and 3.0 for the nodes examined. Chl*a/b* of red maple ranged from 2.4 to 3.3, and from 3.4 to 3.7 in redbud. Carotenoid/chlorophylln ratios significantly decreased from the tip down in all anthocyanic species ($r^2 = 0.79$, 0.62, and 0.63 for sweetgum, red maple, and redbud, respectively; $P < 0.001$ for all species) (Fig. 2, Table 1), but of the acyanic species, only ivy showed significant declines ($r^2 = 0.56$; $P < 0.0001$; $P = 0.18$ and 0.12 for honeysuckle and forsythia, respectively; data not shown). Mean car/chl ratios of the youngest nonred leaves of the anthocyanic species were similar (*c.* 108% of values of mature leaves), although a Tukey–Kramer test indicated car/chl ratios of sweetgum to be significantly higher than those of red maple ($P = 0.0031$), while other species combinations did not significantly differ. In all anthocyanic species, ranges of car/chl ratios of leaf nodes measured were similar (mean of 140% for the youngest leaf node, 100% for the second nonred leaf node).

Gas exchange

The youngest nonred juvenile leaves of sweetgum (third node) had significantly higher photosynthesis under saturating light conditions (A_{sat}) for both absolute and percentage-of-mature values compared with those of red maple and redbud ($P = 0.0003$ for absolute values and 0.0029 for percentage-of-mature values per unit area; $P = 0.0018$ and 0.002 per unit volume, respectively) (Table 1, Fig. 3). Mean A_{sat} (per unit area) observed for the youngest nonred leaves of sweetgum was 11.5 μmol m^{-2} s^{-1} (corresponding to 99% full mature maximum photosynthetic values), while red bud and red maple averaged 6.5 μmol m^{-2} s^{-1} (corresponding to *c.* 60% of mature values). On a per-unit-volume basis, A_{sat} for the first nonred leaf of sweetgum was 66.4 mmol m^{-3} s^{-1} (130% mature photosynthesis), 55.5 mmol m^{-3} s^{-1} for redbud (77.6% of mature), and 38.1 mmol m^{-3} s^{-1} for red maple (81.6% of mature). In fully mature leaves, the three species did not differ significantly with respect to photosynthesis per unit leaf area, which averaged 11 μmol m^{-2} s^{-1} ($P = 0.275$). However, on a per-unit-volume basis, mature leaves of redbud had significantly (*c.* 33%) higher photosynthesis compared with red maple and sweetgum ($P = 0.005$). Mean values for mature leaf A_{sat} on a unit-volume basis were 72.4 (redbud), 46.7 (red maple), and 50.7 mmol m^{-3} s^{-1} (sweetgum). Percentage mature photosynthesis in sweetgum, red maple, and redbud correlated positively with the percentage of mature chlorophyll content (area basis) ($r^2 = 0.53$, 0.98, 0.91, and $P = 0.001$, < 0.0001, < 0.0001, respectively) and leaf thickness ($r^2 = 0.86$, 0.97, and 0.93; $P < 0.0001$ for all species), and negatively with anthocyanin ($r^2 = 0.90$, 0.80, 0.60; $P < 0.0001$ for all species) (Fig. 4).

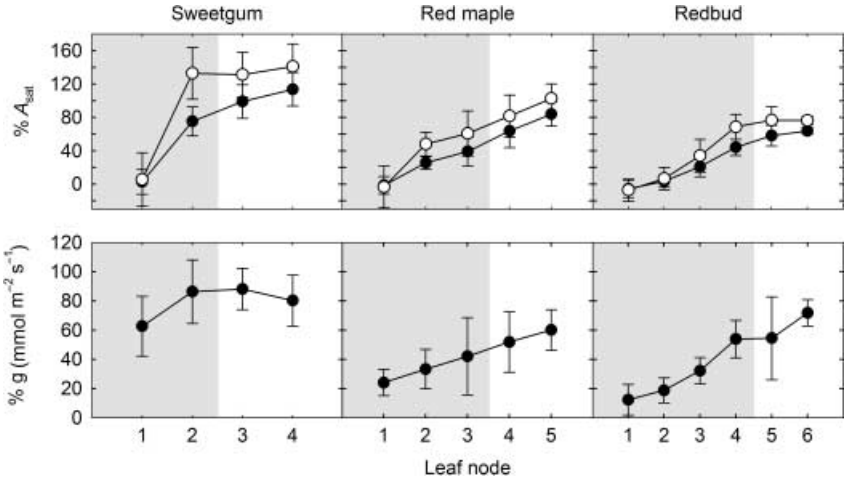

Fig. 3 Percentage maximum photosynthesis (A_{sat}) and leaf conductance of water vapor (g) values by leaf node, expressed as a percentage of mature-leaf values. Shaded areas represent the visible presence of anthocyanin. Illustrated are: percentage photosynthesis expressed as per unit area (closed circles; μmol m^{-2} s^{-1}) and per unit volume (open circles; mmol m^{-3} s^{-1}) and percentage conductance for *Liquidambar styraciflua* (sweetgum), *Acer rubrum* (red maple) and *Cercis canadensis* (redbud). Points represent means of five to 10 replicates ± SD.

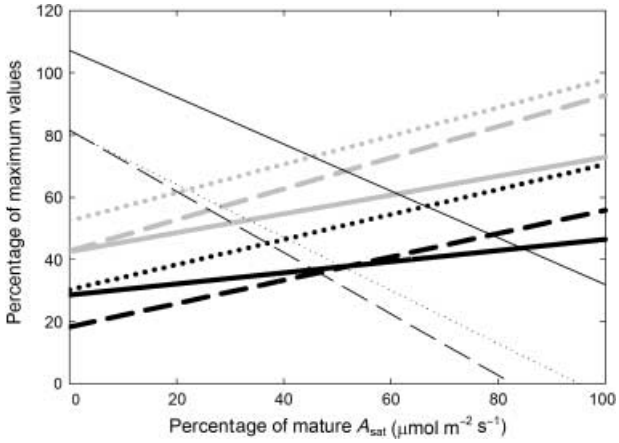

Fig. 4 Relationship between photosynthesis maturation and corresponding changes in chlorophyll per unit area (thick black), leaf thickness (thick gray), and anthocyanin (thin black). Solid lines, *Liquidambar styraciflua* (sweetgum); long dashes, *Acer rubrum* (red maple); dotted lines, *Cercis canadensis* (redbud). Percentage of mature photosynthesis in sweetgum, red maple, and redbud correlated positively with percentage of mature chlorophyll content (r^2 = 0.53, 0.98, and 0.91, respectively) and leaf thickness (r^2 = 0.86, 0.97, 0.93), and negatively with anthocyanin (r^2 = 0.90, 0.80, 0.60). The slopes of these lines were also statistically similar between species. For chlorophyll content, the slopes were 0.179, 0.375, and 0.340 for sweetgum, red maple, and redbud, respectively; for thickness, the slopes were 0.304, 0.500, and 0.393, and for anthocyanin, they were –0.750, –0.985, and –0.775.

Although ANOVA tests showed a significant difference between leaf conductance rates of the three species at the first nonred leaf node (P = 0.0369), the Tukey-Kramer test for means difference did not indicate a significant difference between any two pairs. Mean leaf conductance values for the youngest nonred leaves of the three species corresponded to 143 (sweetgum), 104 (red maple), and 130 mmol m^{-2} s^{-1} (redbud). Similarly, there were no differences among the three species in the percentage of mean maximum leaf conductance relative to mature leaves at the time of anthocyanin disappearance (P = 0.1850), and the youngest, fully green leaves of all species averaged 68% of the mean maximum leaf conductance measured for mature leaves.

Discussion

Photopigment accumulation

In all species examined, anthocyanins were visible until approx. 44% chlorophyll (per unit area), 58% chlorophyll (per unit volume) and 49% of total carotenoid content (per unit area) had developed (Fig. 2). These values did not vary significantly between species. Similar chlorophyll contents have also been reported for developing leaves of *Rosa* sp. and *Ricinus communis*, in which anthocyanic leaves had up to *c.* 50% chlorophyll and carotenoid contents of mature leaves (per unit area) (Manetas *et al.*, 2002). This interspecific similarity in photopigment concentrations observed at the time of anthocyanin disappearance suggests a degree of photosynthetic maturity that no longer requires photoprotection by anthocyanin. Perhaps photopigment concentrations near 50% are low enough to avoid excessive light capture (i.e. beyond that which can be efficiently processed by immature chloroplast and/or anatomical structure), and/or concentrations are high enough to contribute significantly to self-shading and, in the case of the xanthophylls, nonphotochemical quenching. Interestingly, these values also correspond closely with those reported by Lee *et al.* (2003) during anthocyanin formation in a variety of temperate deciduous tree species during autumn leaf senescence. In that study, anthocyanin synthesis was found to be initiated when < 50% chlorophyll still remained, corresponding to < 200 mg chl m^{-2}. Here, we report a similar pattern for a different life and phenological stage, that is, anthocyanins were present only until *c.* 50% mature chlorophyll content was reached in maturing leaves, corresponding also to < 200 mg m^{-2}.

It is notable that, compared with the more constant increase in chlorophyll per unit area during leaf development, chlorophyll per unit volume remained relatively unchanged at *c.* 58% of mature leaf values at all measured nodes for all three species (Fig. 2, Table 1). This suggests that much of the increase in chlorophyll content per unit area observed during maturation may be attributable to mesophyll thickening, rather than to increased intracellular chlorophyll content. This is supported by the observations that increases in chlorophyll per unit area paralleled increases in leaf thickness when plotted against photosynthetic capacity (Fig. 4), and also by the visible elongation of palisade cells during mesophyll thickening (Fig. 1). The maintenance of relatively low chlorophyll content per unit volume (*c.* 58% in young leaves) further supports a decreased emphasis on light capture in younger leaves, with additional chlorophyll likely being added later in development following completion of additional leaf-structural and chloroplast maturation (Smith *et al.*, 1997; Pettigrew & Vaughn, 1998; Niinemets *et al.*, 2004). Although not significant, it should be noted that chlorophyll per unit volume did increase somewhat during development in all species (Fig. 2), indicating the addition of some intracellular chlorophyll.

In addition to decreased anthocyanin concentrations during leaf development, car/chl ratios also significantly declined (Fig. 2), probably reflecting a decreased need for photoprotection through nonphotochemical quenching (NPQ) as leaves matured. These results are consistent with findings reported for both acyanic and anthocyanic juvenile-leafed species (Krause *et al.*, 1995; Barker *et al.*, 1997), but contrast results of others reporting no significant change in carotenoid pools during development, even when juvenile leaves were anthocyanic (Manetas *et al.*, 2002). It has been suggested that species with elevated carotenoids during juvenile phases may develop other photoprotective structures when leaves mature (such as highly reflective layers), which effectively replace photoprotection by

anthocyanins and/or elevated carotenoid pools (Manetas *et al.*, 2002). In all species examined here, leaf maturation was accompanied by gradual whitening (presumably because of the thickening of highly reflective epicuticular waxes) on abaxial surfaces (data not shown). These layers likely function to increase reflectance of incident sunlight on abaxial surfaces of these dorsiventrally asymmetric leaves, which tend to be more light-sensitive than adaxial surfaces (Sun *et al.*, 1996; Smith *et al.*, 1997; Sun & Nishio, 2001). Indeed, in redbud and *Ailanthus altissima* (data not shown), anthocyanin in the abaxial surface remained visible longer than adaxial anthocyanin, supporting the importance of abaxial-specific photoprotection in both juvenile and mature leaves (Hughes & Smith, 2007). The concomitant decline of high car/chl ratios and anthocyanins observed here may therefore represent another degree of coordination leaves exhibit during development – the concerted decline of juvenile-specific photoprotective strategies with the maturation of others more characteristic of fully expanded leaves.

Previous studies have demonstrated that developing chloroplasts generally exhibit higher proportions of chl*b* relative to chl*a* in developing leaves, corresponding with higher proportions of PSII relative to PSI (Pettigrew & Vaughn, 1998). The lag in PSI development during chloroplast maturation corresponds with decreased unappressed thylakoid membrane surface area availability early in chloroplast development, which is where PSI complexes primarily occur (Pettigrew & Vaughn, 1998; Hopkins & Hüner, 2004b). Chl*a* concentrations increase as unappressed membranes increase in number. Consistent with this trend, the anthocyanic species evaluated here had significant increases in chlorophyll *a*/*b* during development (Figs 2, 5; Table 1). However, these values were much lower than those observed for species with acyanic juvenile leaves, with chl*a*/*b* ratios for two acyanic species remaining relatively unchanged during development (Fig. 5). The reduced chlorophyll *a*/*b* ratios in red leaves relative to green are likely the result of the shading effect of anthocyanin. Previous studies have shown that shade both retards growth and developmental rates (thus reducing the rate of chl*a* increase), and decreases the ratio of core relative to light-harvesting complexes (Cui *et al.*, 1991; Jones, 1995; Demmig-Adams, 1998; Hopkins & Hüner, 2004a). This explanation is supported by the observation that redbud, containing significantly less anthocyanin in young nodes than the other two species, had the highest chlorophyll *a*/*b* ratios of the three species, presumably because of less shading (Fig. 2, Table 1). The shading effect of anthocyanin in juvenile leaves has been investigated in greater detail by Manetas *et al.* (2003) using red and nonred variants of *Quercus coccifera*. In addition to lower chlorophyll *a*/*b* ratios, red juvenile leaves of *Q. coccifera* had a suite of other shade characteristics, including thinner leaves and lower concentrations of xanthophyll cycle pigments relative to acyanic variants. Presumably, these differences were the result of shading by anthocyanic layers which, as previously mentioned, reduce incoming sunlight via strong absorption of blue-green light.

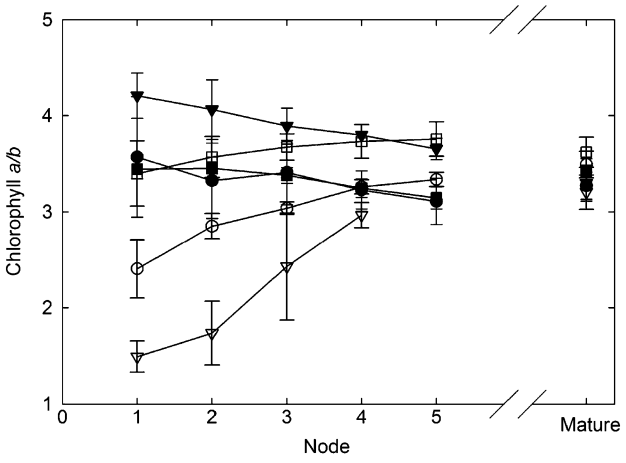

Fig. 5 Tip-down (nodes 1–5) chlorophyll *a*/*b* ratios for species with green developing leaves (closed symbols) and red (open), compared with mature leaf values. Green (acyanic) species include *Hedera helix* (circles), *Forsythia suspensa* (triangles), and *Lonicera japonica* (squares). Red species include *Acer rubrum* (circles), *Liquidambar styraciflua* (triangles), and *Cercis canadensis* (squares). Points represent means of five replicates ± SD.

Leaf structure and gas exchange

Other factors in addition to photopigment content must also be considered when evaluating photon absorption and processing, including leaf (mesophyll) thickness, structural maturation (e.g. spongy and palisade mesophyll differentiation and chloroplast development), leaf conductance of CO_2, and photosynthetic CO_2 fixation (see reviews in Smith *et al.*, 1997; Evans *et al.*, 2004).

A strong relationship was found between leaf thickness and the disappearance of anthocyanin during leaf maturation. On average, anthocyanin loss occurred only when *c.* 80% of mature leaf thickness had been attained (Fig. 2). These values did not vary significantly between species. Photosynthesis (computed on a leaf area basis) also showed a strong, positive correlation with leaf thickness (Fig. 4), most likely because of increases in chlorophyll per unit area and chloroplast development, as well as leaf structural maturation.

To infer structural maturation, chlorophyll content expressed per unit volume may be compared with photosynthesis per unit volume. In red maple and redbud, photosynthesis expressed per unit leaf volume continued to increase through node 5 (Fig. 3), even though chlorophyll content per unit volume did not change significantly (Fig. 2). This suggests that either (or both) chloroplast development was still ongoing (in terms of thylakoid number per granum, membrane complexity, stroma lamellae development, etc.), or that leaf anatomical structure facilitating light distribution and/or CO_2 diffusion was still in the process of maturation. Although

we did not observe chloroplast development in this study, we were able to observe differentiation of mesophyll into palisade and spongy layers (Fig. 1). These structures can be important for enhancing light propagation and processing by distributing absorbed light more uniformly throughout the mesophyll (in the case of palisade cells) and facilitating CO_2 diffusion (spongy) (Vogelmann *et al.*, 1996; Smith *et al.*, 1997). In all species, anthocyanins persisted until palisade cells had elongated to comprise *c.* 40–50% of the mesophyll space (Fig. 1). Although not quantified, air spaces in the spongy mesophyll were also more conspicuous in nodes following anthocyanin disappearance than preceding. This suggests that anthocyanins are particularly important in reducing internal light until leaf anatomy has matured to process it more efficiently.

Paralleling increases in photosynthesis, leaf conductance of CO_2 also increased significantly during development for all species (Fig. 3), with the most rapid increase between leaf nodes occurring in sweetgum. Because mesophyll conductance is a significant factor affecting photosynthetic potential, it is to be expected that stomata would not open fully until leaves are structurally and biochemically mature enough to process increased internal CO_2. This may explain why conductance values in sweetgum approached mature values earlier than red maple and redbud, corresponding to its more rapid structural maturity (Fig. 1).

The increase in photosynthetic carbon fixation facilitated by a mature internal leaf anatomy may also provide a greater sink for dissipating excessive sunlight energy, potentially decreasing the requirement of photoprotective mechanisms such as anthocyanin (Smith *et al.*, 1997). In support of this idea, sweetgum (having the highest photosynthesis in young leaves) lost anthocyanin more quickly during development (i.e. at significantly lower chlorophyll content) than the other two species examined. By the third leaf node, where leaf anthocyanin was no longer visible in sweetgum, photosynthesis per unit area had reached *c.* 99% of full capacity, although only *c.* 138 mg m^{-2} (44% of mature) chlorophyll had developed (Fig. 3, Table 1). In contrast, anthocyanin remained visible until the fourth leaf node in red maple (which had only reached *c.* 60% of full photosynthetic capacity, with *c.* 298 mg m^{-2} chlorophyll), and the fifth node in redbud (*c.* 40% of full capacity, and *c.* 159 mg m^{-2}). The association of rapid structural and photosynthetic maturation with rapid anthocyanin decline provides further evidence for a strong functional coupling among leaf structure, anthocyanin presence, and photosynthetic maturation.

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