BRIEF COMMUNICATION

Effect of transient accumulation of anthocyanin on leaf development and photoprotection of *Fagopyrum dibotrys* mutant

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Abstract

Changes in pigments contents, leaf area, leaf dry mass per unit area (LMA), photosynthetic rate and chlorophyll *a* fluorescence were investigated in developing leaves of *Fagopyrum dibotrys* Hara. mutant. Anthocyanins transiently accumulate below the upper epidermis during leaf ontogeny of this mutant. Red leaves possessed lower Chl content, LMA, photosynthetic rate and apparent carboxylation efficiency than green leaves. However, content of anthocyanins declined and above mentioned parameters increased during further leaf development. In both red and green leaves, chronic photoinhibition did not take place according to variable to maximum chlorophyll fluorescence ratio (F_v/F_m). Red leaves had higher non-photochemical quenching (NPQ) and higher PS 2 efficiency.

Additional key words: chlorophyll fluorescence, green and red leaves, net photosynthetic rate, PS 2 efficiency.

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The juvenile stage of leaf development is the most vulnerable time in the life cycle of a leaf, and anthocyanin accumulation is often present during this stage (Coley and Kursar 1996, Numata *et al.* 2004). The appearance of anthocyanins in leaf tissue is also correlated to stresses such as low temperature in combination with excessive sunlight (Krol *et al.* 1995, Janda *et al.* 1996), UV-B radiation (Ravindran *et al.* 2001, Ambasht and Agrawal 2003), pathogen infection (Hipskind *et al.* 1996), nutrient deficiency (Ulrychová and Sosnová 1970, Kumar and Sharma 1999), herbivore feeding (Kursar and Coley 1992, Dominy *et al.* 2002), and general injury (Stone *et al.* 2001). During protection against such stresses, the anthocyanins can act as antioxidants (Chalker-Scott 1999) and/or as signal that a plant is undergoing stress. The role of anthocyanin pigments during leaf development is photoprotection since red pigments typically accumulate either on one leaf surface or on both, subsequently masking green chlorophyll (Chl) reflectance or assisting red light absorption (Pomar and Barceló 2007). Red leaves absorb from blue to green to yellow radiation (Hughes and Smith 2007). For example, anthocyanins located within the epidermis of jack pine has been found to reduce its sensitivity to photoinhibition at low temperatures by means of radiation attenuation (Krol *et al.* 1995) and reduced risk of photo-oxidative damage to leaf tissue by masking Chl (Feild *et al.* 2001). In addition, anthocyanins in *Cistus creticus* act as a radiation screen and/or antioxidants (Kytridis *et al.* 2008).

Anthocyanin accumulation may also affect other pigment biosynthesis due to the carbon skeleton cost and photosynthesis due to competition between anthocyanins and Chl to capture radiation. Red leaves have been

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reported to possess lower CO_2 assimilation and Chl a/b ratios, and they are generally thinner than green leaves (Manetas et al. 2003, Kyparissis et al. 2007). The maximum photosynthetic rate of red leaves was usually lower compared to green leaves (Lambers et al. 1998).

With the aim to clarify the photoprotective function of anthocyanins, an array of morphologic and photo-synthetic parameters was investigated in $Fagopyrum$ $dibotrys$ mutant over-accumulating anthocyanins during leaf development.

The $Fagopyrum$ $dibotrys$ Hara. is perennial buckwheat, and the rhizomes have been utilized for medicinal purposes in China, Thailand and Nepal. $F.$ $dibotrys$ mutant was introduced by means of γCo^{60} irradiation in 2007 and was utilized from the M2 generation. Experiments were carried out from April to June, 2009, at the research station of the Institute of Medicinal Plant Development (lat 39°47′ N, long. 116°25′ E, alt. 50 m), located in a temperate continental climatic region. $F.$ $dibotrys$ roots were cut into approximately 3 cm long pieces having no more than three sprouts and planted in the greenhouse for approximately 14 d until the third leaf of each individual plant sprouted. Seedlings were then transplanted into fields. Red pigments accumulated on the abaxial leaves of the $F.$ $dibotrys$ mutant while the original (control) plant remained green. The seedlings were approximately 10 cm in height 15 d after transplantation and the leaves of the mutant turned green except for third or fourth apex leaves after three weeks. Three to ten leaves were chosen from control and mutant plants at 15-d intervals until the visible anthocyanins could not be seen by the naked eye. Leaf pigments contents, leaf area, leaf dry mass, net photosynthetic rate, photosystem 2 (PS 2) efficiency, and non-photochemical quenching (NPQ) were examined in 10, 25, 40 and 55-d-old plants.

Leaves were cut into 50 to 100 μm sections by vibratome ($Leica$ $CM1900$, $Leica$ $Microsystems$, Bannockburn, IL, USA). The sections were then mounted on a $Zeiss$ $Axio$ $Imager$ $A1$ microscope ($Carl$ $Zeiss$, Thornwood, NY, USA). Leaf thickness was measured using $AxioVision$ software. Leaf area was analyzed using the $AM300$ portable leaf area meter (ADC $BioScientific$, Hoddesdon, UK). Anthocyanins were extracted in plastic vials containing 5 cm^3 HCl:H_2O:MeOH (1:23:79) in the dark for 24 h at 4 °C. Anthocyanin content was determined spectrophotometrically (UV 2550, $Shimadzu$, Tokyo, Japan) as A_{530} - 0.24 A_{653} (Murray and Hackett 1991). Chl were extracted in 80 % acetone. Chl content was determined by measuring A_{663} and A_{646} nm and calculated according to Lichtenthaler (1987). For determination of leaf dry mass per unit area (LMA) selected leaf samples were dried at 80 °C for 24 h.

The light response curves were measured by LI-6400 open photosynthesis system (LI-COR, Lincoln, NE, USA) fitted with a red-blue LED light source and a CO_2 mixer on 40 and 55-d-old plants. Data were collected between 08:00 and 11:30. Temperature and relative air humidity inside the chamber were 30 ± 4 °C and 30 ± 5 %, respectively, CO_2 concentration about 400 μmol mol^{-1}, and irradiance was greater than 1 500 μmol m^{-2} s^{-1}. Prior to measurement, plant samples were allowed to fully adapt for 30 min inside the leaf chamber to activate leaf conductance. The maximum photosynthetic rate (P_{Nmax}) was computed according to the equation by Prado and Moraes (1997).

Chlorophyll a fluorescence was determined on the third fully exposed leaves from the apex using the LI-6400 system fitted with a 6400-40 leaf chamber fluorometer. Variable to maximum fluorescence ratio (F_v/F_m) was measured in the predawn and calculated as (F_m - F_0)/F_m (Maxwell and Johnson 2000). Minimum fluorescence (F_0) was determined under dim light (< 0.5 μmol m^{-2} s^{-1}) after 15 min of dark adaptation. A saturating flash of 6 000 μmol m^{-2} s^{-1} of 0.8 s duration was used to determine maximum fluorescence (F_m). Leaves were kept in a chamber with approximately 1 800 μmol m^{-2} s^{-1} actinic light for determination of $F_0′$ and $F_m′$ during a period between 08:00 and 13:30. PAR was reprogrammed after every cycle and lowered from 1800 to 0 μmol m^{-2} s^{-1}. A saturation flash of 6 000 μmol m^{-2} s^{-1} for 0.8 s was provided to obtain $F_m′$ of light-adapted leaf. $F_0′$ was measured after gradually reducing irradiance and using the far-red (FR) of 7 μmol m^{-2} s^{-1} for 0.6 s. The experiments were continued in steps using the same cycle of flashes and FR irradiation for 4 min intervals. The PS 2 photochemical efficiency in the light-adapted state was calculated according to Genty (1989) as $\Delta F/F_m′ = (F_m′ - F_0′)/F_m′$ and NPQ was calculated according to Bilger and Björkman (1990) as NPQ = $(F_m - F_m′)/F_m′$.

One-way $ANOVA$ was carried out to analyze the significance of differences among treatment group means for the measured parameters ($Systat$ $Software$, Chicago, IL, USA).

The lower epidermis and mesophyll cell layer of the red leaves exhibited reddening after transplantation. The upper epidermis remained green during this period. Slight anthocyanin accumulations were observed within the veins of green leaves (data not shown). Red leaf anthocyanin accumulation remained visible to the naked eye for approximately 60 d, and the veins of green leaves maintained a pale red color throughout the experimental period. Red leaves contained considerably more anthocyanins than green leaves (P < 0.01) at every stage (Table 1). Anthocyanin accumulation in red leaves was three times higher than in green leaves during the first stage. Thereafter the red coloring in leaves became invisible to the naked eye, even red leaves had approximately 23 % higher anthocyanins accumulation than green leaves.

Leaf area and thickness increased gradually during growth periods (Table 1), indicating that they were enlarging as they progressed from young to mature states.

Table 1. Anthocyanin content (n = 6 - 10), the leaf area (n = 6 - 10), leaf thickness (n = 10), LMA (n = 10), chlorophyll content and Chl a/b ratio (n = 5), maximum photosynthetic rate (P_{Nmax}) (n = 6), apparent carboxylation efficiency (P_N/c_i) (n = 6), maximum photochemical efficiency of dark-adapted leaves (F_v/F_m) (n = 6), maximum photochemical efficiency of light-adapted leaves ($\Delta F/F_m'$) and non-photochemical quenching (NPQ) of red and green leaves of mutant and control plants under 1500 μmol m^{-2} s^{-1}. Means ± SD. Different letters indicate significant differences (P < 0.01).

Parameters		10-d-old	25-d-old	40-d-old	55-d-old
Anthocyanin content	green leaves	0.598 ± 0.100b	0.638 ± 0.096b	0.644 ± 0.082b	0.645 ± 0.087b
[mg g^{-1}(f.m.)]	red leaves	1.631 ± 0.079a	1.049 ± 0.080a	0.860 ± 0.088a	0.822 ± 0.099a
Leaf area	green leaves	4135 ± 357b	5302 ± 428b	6360 ± 678b	6500 ± 726b
[mm^2]	red leaves	5637 ± 469a	6558 ± 556a	7800 ± 430a	7850 ± 680b
Leaf thickness	green leaves	0.218 ± 0.005a	0.228 ± 0.006a	0.231 ± 0.012a	0.233 ± 0.009a
[mm]	red leaves	0.198 ± 0.002b	0.204 ± 0.006b	0.214 ± 0.007a	0.218 ± 0.014a
LMA	green leaves	6.560 ± 0.059a	7.450 ± 0.167a	7.895 ± 0.226a	7.900 ± 0.247a
[mg cm^{-2}]	red leaves	4.950 ± 0.168b	6.710 ± 0.106b	7.400 ± 0.213a	7.535 ± 0.163a
Chl $a+b$ content	green leaves	2.048 ± 0.034a	2.900 ± 0.136a	2.932 ± 0.099a	3.182 ± 0.056a
[mg g^{-1}(f.m.)]	red leaves	1.550 ± 0.237b	2.329 ± 0.117b	2.561 ± 0.031b	2.981 ± 0.064b
Chl a/b	green leaves	3.771 ± 0.197b	3.589 ± 0.224b	3.513 ± 0.233b	3.510 ± 0.243b
	red leaves	4.281 ± 0.108a	3.494 ± 0.133b	3.380 ± 0.009b	3.360 ± 0.080b
P_{Nmax}	green leaves	23.07 ± 0.545a	25.08 ± 0.665a	26.86 ± 0.422a	26.87 ± 0.854a
[μmol m^{-2}s^{-1}]	red leaves	17.78 ± 1.121b	20.09 ± 1.488b	26.10 ± 0.528a	26.22 ± 0.750a
P_N/c_i	green leaves	0.080 ± 0.006a	0.104 ± 0.021a	0.105 ± 0.007a	0.108 ± 0.010a
[mol m^{-2}s^{-1}]	red leaves	0.066 ± 0.008b	0.080 ± 0.008b	0.100 ± 0.009a	0.101 ± 0.014a
F_v/F_m	green leaves	0.822 ± 0.005a	0.836 ± 0.002a	0.851 ± 0.004a	0.850 ± 0.005a
	red leaves	0.801 ± 0.002b	0.831 ± 0.002a	0.848 ± 0.006a	0.847 ± 0.005a
$\Delta F/F_m'$	green leaves	0.410 ± 0.006b	0.474 ± 0.014a	0.509 ± 0.007a	0.538 ± 0.007a
	red leaves	0.454 ± 0.201a	0.420 ± 0.016b	0.482 ± 0.007b	0.513 ± 0.007b
NPQ	green leaves	2.335 ± 0.676b	2.524 ± 0.084b	2.807 ± 0.171b	2.972 ± 0.170b
	red leaves	2.768 ± 0.109a	2.974 ± 0.410a	3.294 ± 0.113a	3.494 ± 0.133a

Red leaf area was considerably larger than green leaf area during the initial development stage; green leaf thickness and LMA were considerably greater than in red leaves during the first and second stage. Maksymowych (1973) observed that initial leaf thickness is established during early leaf development following a phase of rapid thickening. Thicker leaves allow a greater photosynthetic apparatus per unit leaf area while broader, thinner leaves can intercept more photosynthetically active radiation (PAR; White *et al*. 2005). This may be the cause why red leaves had larger overall leaf area compared to green leaves.

Leaf Chl content increased in conjunction with an increase in leaf area, and it was considerably higher in green leaves as compared to red leaves (Table 1). Similar pattern of Chl content have also been reported for developing leaves of *Rosa* sp. and *Ricinus communis* where leaves with high anthocyanin contents had up to 50 % greater Chl content compared to mature leaves (Manetas *et al*. 2002). Merzlyak (2008) also observed that leaves with anthocyanins contained low Chl content. During leaf development, young leaves require high photodissipative capacity (Krause *et al*. 1995). Therefore, young red leaves containing low Chl and high anthocyanin contents can alleviate these requirements due to lessened Chl excitation pressure (Manetas *et al*. 2002).

With regard to the mutant of *F. dibotrys* that contained low Chl content and thin leaves, this may be the result of an adaptive mechanism triggered by excessive sunlight (>1200 μmol m^{-2} s^{-1}). Larger red leaf area in comparison to green leaf area may be the result of competition of anthocyanins with Chl for light absorption (Merzlyak *et al*. 2008). Red leaf area spread may therefore be the result of compensatory adaptation to capture greater amounts of sunlight.

The visible anthocyanin of red leaves dramatically decreases while Chl increases during development, but Chl content and leaf LMA and thickness were still lower in red leaves than in green leaves. The red leaves growth was probably retarded by the anthocyanin accumulation (Jones 1995, Hopkins and Hüner 2004).

Anthocyanins always absorb blue to green to yellow radiation, therefore, higher anthocyanins accumulation in red leaves essentially eliminate the radiation absorbed by chlorophyll and so provide protection against photoinhibition especially in young leaves (Manetas 2006).

The lower Chl a/b ratio in red leaves than in green leaves indicates decrease in core complex (exclusively Chl a) relative to the light-harvesting (both Chl a and Chl b) complex (Hopkins and Hüner 2004).

The P_{Nmax} and apparent carboxylation efficiency were higher in green leaves compared to red leaves during leaf

development (Table 1). Previous study showed that red leaves possessed a lower area-based photosynthetic rate, apparent carboxylation efficiency, and LMA than green leaves due to shade acclimation (Lichtenthaler *et al.* 2000). The red mutant of *F. dibotrys* was partly consistent with these characteristics. Moreover, anthocyanins retarded leaf growth and compete with Chl for light. This is consistent with lower LMA, thickness, and photosynthetic rates in red leaves throughout development.

Our results suggested that photoinhibition did not take place in either red or green leaves because F_v/F_m remained approximately 0.8 at all times (Table 1) while it increased during leaf development as in European beach (Čaňová et al. 2008). The F_v/F_m in red leaves was slightly lower than in green ones. The phenomenon was also found in red leaves of *Prunus cerasifera*, which possessed slightly lower F_v/F_m values compared to green leaves (Kyparissis *et al*. 2007). Leaf $\Delta F/F_m'$ values were higher in red than in green leaves during the first stage but they decreased during succeeding stages, indicating that a portion of light absorbed by anthocyanins in red leaves was not used in photosynthesis (Table 1). This is

consistent with the low P_N. Based on previous reports (Merzlyak *et al.* 2008) anthocyanins accumulated in red leaves possessed a protective function during leaf development. Certain studies have also shown high amounts of anthocyanins in young red leaves of *Anthocephalus chinensis* and *Litsea pierrei* possessing low diurnal photoinhibition (Cai *et al*. 2005).

NPQ increased gradually for both leaf types during development (Table 1). However, NPQ was higher in red than in green leaves, which reveals that red leaves dissipated a greater amount of light energy as heat than green leaves in order to protect the photosynthetic apparatus. Also previous study showed that red leaf NPQ was higher compared to green leaf NPQ (Li *et al*. 2009).

In conclusion, red leaves with greater anthocyanin accumulation below the upper leaf surface likely impose a limitation to leaf development but possess a photoprotection capacity. Further research is required to elucidate the intrinsic mechanism of anthocyanin accumulation and the photosynthetic properties of *F. dibotrys* mutant.

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