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Anthocyanin-dependent anoxygenic photosynthesis in coloured flower petals?

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Chlorophyllless flower petals are known to be composed of non-photosynthetic tissues. Here, we show that the light energy storage that can be photoacoustically measured in flower petals of *Petunia hybrida* is approximately 10–12%. We found that the supposed chlorophyllless photosynthesis is an anoxygenic, anthocyanin-dependent process occurring in blue flower petals (ADAPFP), accompanied by non-respiratory light-dependent oxygen uptake and a 1.5-fold photoinduced increase in ATP levels. Using a simple, adhesive tape stripping technique, we have obtained a backside image of an intact flower petal epidermis, revealing sword-shaped ingrowths connecting the cell wall and vacuole, which is of interest for the further study of possible vacuole-related photosynthesis. Approaches to the interpretations of ADAPFP are discussed, and we conclude that these results are not impossible in terms of the known photochemistry of anthocyanins.

Most white or coloured flower petals of higher plants are known to be composed of chlorophyllless, non-photosynthetic tissues. In some species, including *Petunia hybrida*¹, only a residual quantity of chloroplasts or chlorophyll can be revealed in petal tissues and conventionally seems to have no physiological significance.

In the study whether the tissue is photosynthetic or not, researchers are restricted by a limited number of direct methods. Thus, the most widely applied method for the detection of photosynthesis is a radiocarbon method or a simple measurement of carbon dioxide assimilation. These methods work, however, only if the photosynthesis is oxygenic, as anoxygenic photosynthesis is not associated with CO₂ or O₂ exchange. However, anoxygenic photosynthesis still converts light energy to that of the of phosphoanhydride bonds of ATP. In this connection, the term “anoxygenic photosynthesis” is commonly accepted as for a number of bacteriochlorophyll-containing bacterial species possessing a cyclic electron transport (CET) in their intracytoplasmic membranes², for example. In the green tissues of higher plants, anoxygenic photosynthesis is usually found to function through CET in the thylakoid membranes^{3,4}.

Unfortunately, photoacoustic (PA) spectrometry is the only direct method for revealing and studying CET (as anoxygenic photosynthesis). The PA method is based on the measurement of acoustic signals that are excited in test samples using a modulated light. If photochemistry is not occurring in a sample, the absorbed light is converted to heat, providing the photothermal component of the PA-signal with an efficiency of 100%. Otherwise, the light energy becomes partially unavailable for conversion as it is stored as photochemical products^{5,6}. In this case switching on a strong background (non-modulated) light saturates the photochemistry, and the acoustic signal amplitude increases with a value equal to that of the energy storage (ES). Such an increase occurs only when the photobaric component is eliminated^{5,7}. Considering the kinetic and spectral parameters of the PA-signal, a nonzero ES value provides an incontrovertible and direct evidence of either oxygenic or anoxygenic photosynthesis^{5–7}.

However, no studies have been reported that evaluate ES in chlorophyllless flower petals due to the belief that there is an absence of photosynthesis as a consequence of the absence (or insufficient presence) of chlorophyll and chloroplasts in these tissues.

A variety of specific physiological and growing photo-dependent effects can be observed during the flowering of higher plants. Thus, it is commonly known that the flowers of heliotropic plants track the sun across the sky to achieve the maximum illumination of their petals. The pollinator attraction⁸ and heat-induced growth promotion⁹ hypotheses which have been proposed to explain floral heliotropism cannot explain the fact that heliotropism is a response to blue light¹⁰.

Light affects anthocyanin level in flower petals as well as induces morphological alterations in anthocyanin-accumulating vacuoles¹¹.

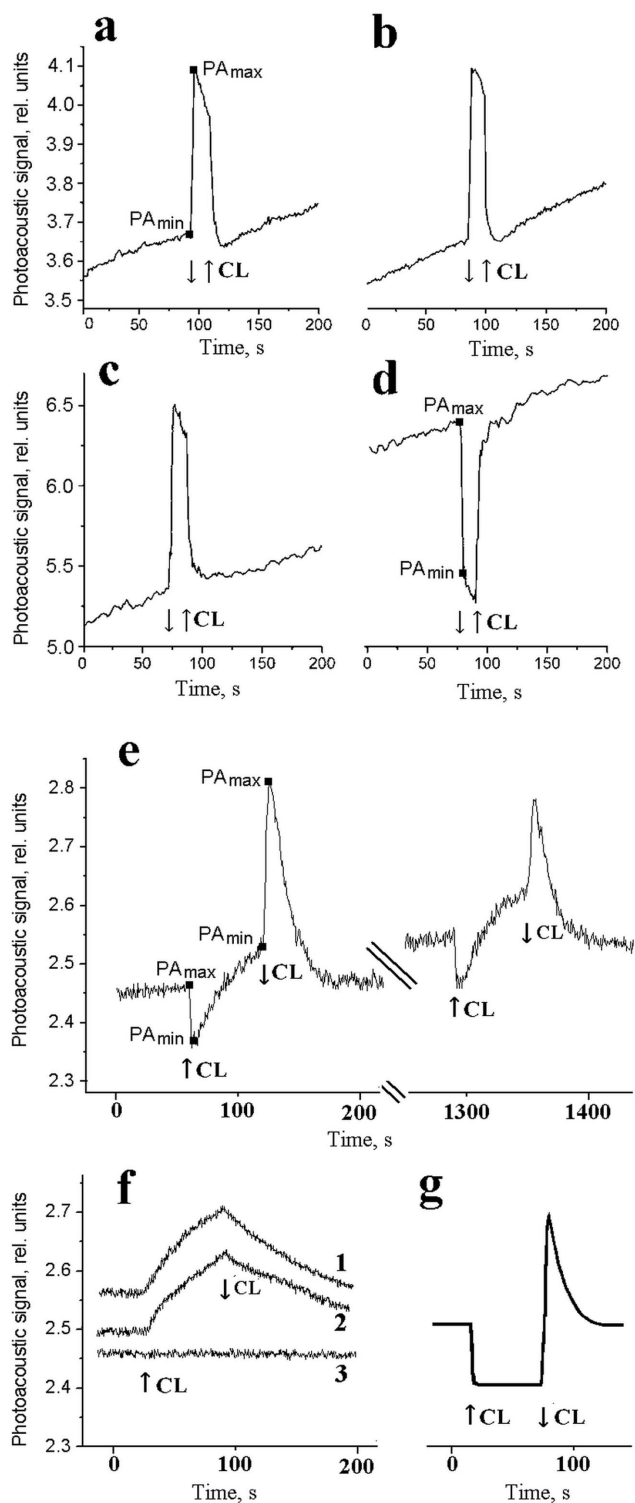


Figure 1 | Time resolved photoacoustic (PA) signal of *P. hybrida* tissues. (a–d) in response to 15-s dark continuous test pulse (dark CTP) interrupting strong continuous light (white LED, 1800 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$). (a, b) Blue flower petals. (c, d) Green leaves. (a, c) 40 Hz frequencies of the modulating light. (b, d) 280 Hz frequencies. Instantaneous light intensity of the modulating light – 36 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ (white LED), duty factor – 50%. There was no response received from the white flower petals (not shown). The observed positive trend of the baseline is possibly caused by evaporation effects (See text). For the blue petunia flower petals at 40 Hz ES = 11.0 \pm 1.5% (a) and at 280 Hz ES = 11.5 \pm 1.7% (b). For petunia green leaves at 280 Hz ES = 12.7% (d) and for petunia green leaves at 40 Hz (c) ES cannot be calculated because of presence of the photobaric

component. (e–g) in response to 60 s continuous test light pulse (light CTP: white LED, 900 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$). The 1-ms excitation pulses of the modulating light were applied with a period of 25 ms and instantaneous light intensity 900 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$. (e) Blue flower petals. (f) Wet blue paper (curve 1), white *P. hybrida* flower petals (curve 2) and dry blue paper (curve 3). (g) Fast PA signal response (true photosynthetic response) of the blue flower petals was deduced by amplitude-normalised subtraction of the slow PA signal of the white flower petals or wet paper from that of the combined fast/slow PA signal which was generated by blue petals (given schematically). An example of a typical log file (*.xls) corresponding to the transient shown in Fig. 1e is available as Supplementary Data 1. For the blue petunia flower petals in this conditions (short, strong light pulse), when the continuous light was switched on (at 65 s), ES = 4.5% and when it was switched off (at 125 s), ES = 9.9% (e). Downward- and upward-pointing arrows, respectively, show switching the strong continuous light (CL) off and on. ES was calculated as $(PA_{\text{max}} - PA_{\text{min}})/PA_{\text{max}}$, where PA_{max} and PA_{min} are maximal and minimal PA amplitude values, respectively, measured immediately before and after (or in a reverse order) switching background light on/off.

This light-dependent behavior of anthocyanin-coloured flowers cannot be explained within the pollinator-attraction hypothesis but seems to be in accordance with the proposed antioxidative protective role¹² of these pigments. However, reactive oxygen species (ROS) are generated mainly as by-products of photosynthesis in chloroplasts or of respiration in mitochondria. Chloroplasts are absent or almost absent from flower petals. Regarding respiration, ROS must be scavenged by anthocyanins before these antioxidants have been transported from the cytoplasm¹³, begging the question as to what the colored anthocyanins do when they are stored in the vacuole. Moreover, the increase of anthocyanins obviously results in additional light absorbance and heating that, in turn, can have the opposite effect: a stress-induced increase of ROS production. In general, one can agree with Hatier J.-H. and K. Gold¹³, that “the fate of these absorbed quanta is unknown...”, stating that the physiological significance of anthocyanins in flower petals is not completely understood.

The photoacoustic studies in the present work demonstrate that the light energy that is absorbed by blue chlorophyllless *P. hybrida* flower petals is partially utilised for the photochemical processes (ES > 0), but the evolution of oxygen was not revealed by gas-exchange measurements. We found that the respective anthocyanin-dependent, anoxygenic photosynthesis in flower petals (ADAPFP) is accompanied by a photoinduced increase in the ATP level. In seeking potential ADAPFP-related subcellular structures, we developed a simple adhesive-tape stripping technique, that was used to obtain a backside image of an intact monolayer of flower petal epidermis, revealing sword-shaped ingrowths, that connect cell wall with vacuole.

Results

The light energy storage and photoacoustic signal response of a *P. hybrida* blue flower petals to strong continuous white light. When *P. hybrida* blue flower petals were adapted to strong continuous light (white LED, 1800 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) for 3 min, the amplitude of their PA-signal induced by weak white modulating light (white LED, 40 or 280 Hz, instantaneous light intensity 36 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, light duty factor 50%) exhibiting a clear response to switching dark continuous test pulse (CTP) for 15 s (Fig. 1a–b). In this case the studied modulating frequencies (40 and 280 Hz) provided an increase in the PA-signal (ES) by 11.0 \pm 1.5 and 11.5 \pm 1.7% (mean values of 8 replicates \pm SD), respectively. This experiment could be performed using a traditional “light CTP” (the term that is used here instead of “saturating light pulse”) scheme when the PA signal of dark-adapted (only under a weak modulating light) photosynthetic tissue is studied in response to switching strong



continuous light on for a few seconds. If green leaves are tested, this scheme works well in demonstrating that the PA signal response is reversed 100% from that of the “dark CTP” scheme; however, in this study of flower petals, the traditional scheme was rejected because our preliminary experiments resulted in a strongly decreased response ($ES < 2\%$) to light CTP. This decreased ES is possibly due to the proposed ADAPFP requiring a few minutes under strong light to become fully operable.

In contrast to flower petals, the green leaves *P. hybrida* revealed another (well-known and expected) behaviour of PA signal response under different modulating frequencies (40 and 280 Hz; Fig. 1c–d). At 40 Hz, dark CTP results in a PA-signal increase, but at 280 Hz it results in a decrease ($ES = 12.7\%$). The conventional interpretation of the photoacoustic activity of chlorophyll-containing tissues assumes that at low frequencies (up to 100 Hz) the PA signal reflects primarily its “photobaric” component which is currently mostly explained in terms of pulsed photosynthetic oxygen evolution. The photobaric component is in antiphase with the photothermal component. At higher frequencies (>150 Hz), the evolution of oxygen becomes continuous and does not contribute to the PA signal allowing the photothermal component to dominate⁵. Thus, when dark CTP interrupts strong continuous light, photosynthesis exits saturation mode, and the efficiency of the oxygen evolution under modulating light and consequent PA signal increase. Under the same conditions, but at high modulating frequencies in which the photobaric component is absent, the absorbed light energy is converted to chemical energy more effectively, and the PA signal decreases.

Considering this interpretation, the resulting data raise the following question: why does the PA signal of flower petals demonstrate a photobaric-like response at both low and high frequencies? There are only two possible explanations for this phenomenon:

- (1) Flower petals possess fast light-dependent O_2 or CO_2 evolution or uptake – the photobaric component exists at 280 Hz. However, the existence of gas-exchange processes that are characterised by such a fast kinetics in response to light pulse is difficult to comprehend.
- (2) In contrast to the usual chlorophyll-dependent photosynthesis, the proposed anthocyanin-dependent photosynthesis has higher quantum efficiency at high light intensities, which saturate photosynthesis in green leaves. In other words, when light intensity increases from low or moderate levels to a high level, the intensity of photosynthesis decreases in leaves but increases in flower petals. As a result, the PA signal response in flower petals to strong light switching on and off will be reversed compared with those of green leaves. Therefore, in this study, if the photobaric component is absent, the PA signal of flower petals will be increased in response to a dark pulse at 40- and 280-Hz modulating frequencies. The indirect evidence in support of this explanation is based on the above data demonstrating that the proposed ADAPFP most likely requires a few minutes under strong light to become fully operable.

Considering the second explanation, it is not surprising that when the light CTP continued for a longer time (60 s), the fast PA signal response of blue flower petals demonstrated a weak ($ES = 4.5\%$) decrease when the continuous test light was switched on and a strong ($ES = 9.9\%$) increase when it was switched off (Fig. 1e). A fast PA signal response was not revealed for white *P. hybrida* flower petals (Fig. 1f, curve 2), suggesting the role of anthocyanins in light energy storage in flower petals.

Most of the experimental data presented in this study were obtained using blue petunia flowers and white light as this light is most similar to natural daylight. Nevertheless, additional photoacoustic measurements were performed using blue and red petunia flowers as well as blue and red light to perform a rough reciprocal estimation of the spectral PA response.

As shown in Tab. 1, no any significant ES values were found when using red petunia flower petals under either blue or red light. Very low ES values were revealed in blue petunia flower petals under blue light; this result was expected because blue quanta are not absorbed by blue anthocyanins. The ES value was significant in blue flower petals when measured under red light and the mean value slightly exceeded that when measured under white light (12.2 ± 1.4 and $11.5 \pm 1.7\%$ respectively). Clearly, only the red light component is utilised in photochemical reactions when blue petals are exposed to white light.

The experimental data in Fig. 1e, f were obtained using the traditional light CTP scheme at the same average intensity of modulating light, as in the previous experiments ($36 \mu\text{mol photons m}^{-2} \text{s}^{-1}$); however, the duty factor of the applied modulating pulses was 4% at a high instantaneous intensity ($900 \mu\text{mol photons m}^{-2} \text{s}^{-1}$), whereas traditional photoacoustic experiments are performed using a duty factor of 50% at a low light intensity. In our preliminary studies with *P. hybrida* flower petals, such a “short pulse” technique produced a sufficiently higher ES (8–10%) compared to that of the commonly used “long pulse” method ($ES < 2\%$); this result is most likely due to the high instantaneous light intensity of the pulse, adapting the proposed ADAPFP to be more efficient. It should be noted here that the “short pulse” technique can be applied only when the PA signal is processed using fast Fourier transform instead of the commonly used lock-in amplifier as described in our previous work¹⁴.

Along with the fast response, the slow PA signal response was observed gradually increasing during periods when strong continuous light was switched on (Fig. 1e, f). To elucidate the nature of this slow response, the blank experiments were performed using the same procedure but with a wet or dry blue coloured paper instead of flower petals. The slow PA signal response was observed in both the wet paper (Fig. 1f, curve 1) and flower petals (Fig. 1e and 1f, curve 2), whereas the dry paper produced a flat signal, indicating that the signal was unaffected by the CTP (Fig. 1f, curve 3). Therefore, slow PA signal response can most likely be attributed to evaporation effects.

The results of the above photoacoustic studies demonstrate that blue flower petals of *P. hybrida* are able to light energy storage ($ES > 0$) which can be effectively measured using only a dark CTP experiment scheme and/or a “short pulse” technique. The proposed ADAPFP can not be ascribed to residual chlorophyll which is contained in blue petals of *P. hybrida* in too low concentrations, which are comparable with those found in white petals where the light energy storage was not revealed. The chlorophyllless nature of this process is also supported by its specific features distinguishing it from chlorophyll photosynthesis; the absence of saturation under strong light and the requirement of a long lag period to achieve sufficient light energy storage.

Photoacoustic signal of *P. hybrida* flower petals in the millisecond time domain. From the point of view of understanding the mechanism of light energy storage in flower petals, the value of delay time

Table 1 | Light energy storage (ES) photoacoustically measured in the blue and red *P. hybrida* flower petals under blue and red light. Both the continuous ($1800 \mu\text{mol photons m}^{-2} \text{s}^{-1}$) and modulating ($36 \mu\text{mol photons m}^{-2} \text{s}^{-1}$) lights were applied together using the same colour. Dark CTP of 60 s duration

Object	Energy storage measured under lights of different colours. Mean values of 8 replicates \pm SD	
	Blue (blue LED, $\lambda_{\text{max}} = 470 \text{ nm}$)	Red (red LED, $\lambda_{\text{max}} = 640 \text{ nm}$)
Blue petunia flower petals	1.1 ± 0.4	12.2 ± 1.4
Red petunia flower petals	0.9 ± 0.3	<0.3

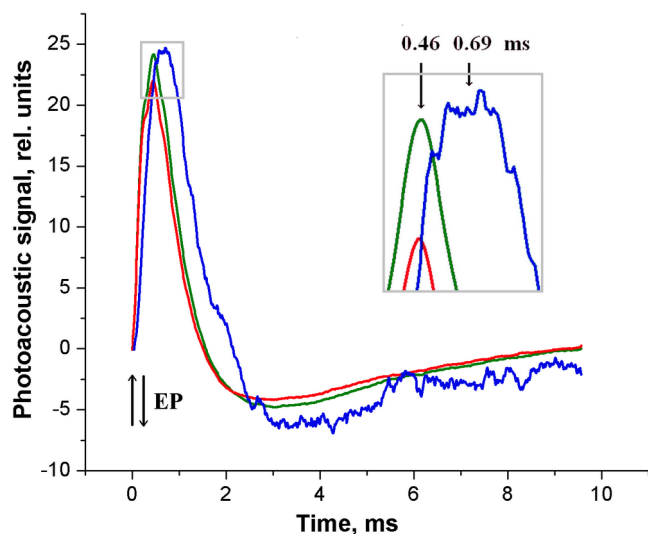


Figure 2 | Shape and time delay of the differential (light/dark) photoacoustic (PA) spike in the millisecond time domain excited in *P. hybrida* flower petals. PA signal was excited by a 0.15-ms light pulses (white LED, 900 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, period 10 ms) and averaged PA-spikes were calculated from 100 individual spikes detected during 1 s before (red curve) and 1 s after (green curve) continuous light (white LED, 900 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$) switching off. The differential PA-spike component (blue curve) generated in response to the light switching off was calculated by subtraction of these two averaged signals and was multiplied by $10\times$ to be normalized approximately near their amplitude maximums. Excitation light pulse is labeled as $\uparrow\downarrow\text{EP}$. Downward arrows descending to the selected area point the time delays of the amplitude maximums from the beginning of the excitation pulse. For details see Supplementary Fig. S2.

attributable to the light-dependent PA-signal response is of great importance.

Direct evaluations of the differential PA signal shape were calculated by the subtraction of two signals that were measured before and after continuous strong light switching, each averaged over 100 individual pulses (Supplementary Fig. S1). The results demonstrate that the differential signal (photodependent response) has a short time delay to the amplitude maximum of less than 0.7 ms (Fig. 2, blue curve). For comparison, the same parameter calculated for the photobaric component of green leaves is approximately 3 ms reflecting the photosynthetic oxygen evolution¹⁵. However, this delay for

the flower petals is too low to be ascribed to gas evolution, and therefore, the obtained differential signal is a time function of the energy storage, indicating some unknown rapid photochemical process which is competing with photothermal dissipation to utilise light energy.

Photoinduced changes of ATP and ADP levels in flower petal epidermis. The ATP level in blue *P. hybrida* flower petal epidermis increased 1.5 times when measured after 30 s of strong light illumination (Fig. 3a), whereas the concurrent ADP level decreased 1.3 times. The ATP level subsequently measured at 60 and 180 seconds of the illumination, and showed a weak decrease. Similar ATP kinetics were observed in experiments on the light excitation of chlorophyll photosynthesis in the green algae *Ankistrodesmus braunii* and chloroplasts from *Pisum sativum*, where the local maximum at 20–30 s was followed by a weak decrease in the ATP level¹⁶. The corresponding ATP/ADP ratio changes in the petal tissues were more strongly pronounced. In contrast to that of blue flower petals, the epidermis of white flower petals did not respond to strong continuous light switching on (Fig. 3b), clearly resulting from that the anthocyanins are necessary here for the development of the photodependent changes of phosphorylation.

Photodependent changes of oxygen levels induced within an airtight chamber in the presence of flower petals. As indicated in Tab. 2, the equilibrium (see the Methods section) oxygen level in a temperature-controlled airtight chamber at $25 \pm 0.5^\circ\text{C}$ in the presence of blue and white flower petals (as well as green leaves) in the dark decreased after 1 h of incubation from 20.8 to 15–16% that was expected considering respiration in petal tissues. When blue (but not white) flower petals were exposed under white light (900 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$), the oxygen level decreased to a sufficiently lower value of 13.1%, possibly indicating the accompanied photorespiration-like activity, but not the photoinduced stimulation of respiration, as anthocyanin-dependent respiration is improbable. In any case, this result demonstrates, at least, that the proposed photosynthetic process is an anthocyanin-dependent, anoxygenic photosynthesis in flower petals (ADAPFP). This conclusion is more clearly evident when comparing the results with those of green leaves (Tab. 2), as these tissues support oxygenic photosynthesis and maintain the oxygen at the initial level of ambient air or even slightly higher (22.2%). Such a small O_2 increase is due to the Warburg effect¹⁷ establishing the O_2 compensation point of C3 plants at approximately 23–25%¹⁸, which is additionally decreased by tissue respiration in an airtight chamber.

It should be noted here that the airtight chamber technique, instead of widely used gas flow exchange methods, was applied in

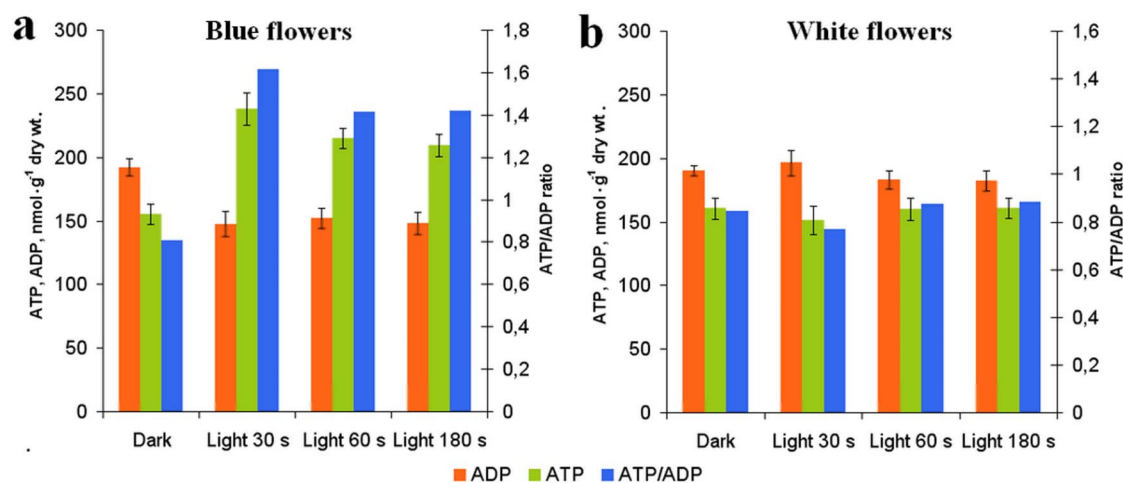


Figure 3 | ATP and ADP levels in the blue (a) and white (b) *P. hybrida* flower petals after 30 min of dark adaptation or after 30- 60- and 180-s strong (900 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, 2700 K°) light switching on. Error bars = SD; $n = 8$.



Table 2 | Photo-dependent changes of the oxygen levels induced by white light (900 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$, 30 min) within an airtight chamber in the presence of flower petals or green leaves of *P. hybrida*

Sample material	O ₂ , % v/v, means of Mean values of 8 replicates \pm SD
Ambient air	20.8 \pm 0.1
Blue flower petals in darkness	15.8 \pm 0.2
Blue flower petals under white LED*	13.1 \pm 0.2
White flower petals under white LED*	15.4 \pm 0.2
Green leaves in darkness	15.0 \pm 0.2
Green leaves under white LED*	22.2 \pm 0.2

this work, to (at least roughly) compare the leaf and flower petal tissues. Thus, the O₂ compensation point is much less dependent than the net O₂ evolution¹⁸ on the chlorophyll content per total mass (activity) of respiratory tissue and on other physiological plant properties. Therefore the results that were obtained using the applied airtight chamber technique allow for a high level of confidence in the conclusion that the proposed chlorophyllless photosynthesis is not related to oxygen evolution despite its ability to be masked by the photoinduced stimulation of respiration.

Microstructures connecting the cell wall and vacuole in flower petal epidermis. Assuming that the proposed ADAPFP is located in vacuoles or in the vacuolar membrane, the adjoining structures are of special interest. In our work peculiar structures, previously not described in the scientific literature (resembling “jigsaw pieces” or “stitches”) were revealed using simple light microscopy in the *P. hybrida* petal epidermis as ingrowths from the cell wall toward the centre of cell. However, in this case, a more detailed picture could not be obtained when studying living cells due to the interference of overlying parts of vacuoles and of underlying cells. In the present research the flower petal epidermis of *P. hybrida* was isolated using a specially developed, simple adhesive-tape stripping technique and was studied by light microscopy using HeliconFocus software allowing for the creation of a single completely focused image and 3D-model from several partially focused images by combining the focused areas. The combined image clearly shows that the ingrowths of the epidermal cell wall are terminated by widenings and are adjoined to vacuoles, much like clavate corpuscles (Fig. 4). As shown using the 3D model compiled in the animated *.gif file (Supplementary Video), these ingrowths are sword-shaped and stretched in height to approximately one half of the vacuole height. The obtained morphological data provide a strong argument for the further investigations of these unusual structures.

Discussion

The results of this study indicate that coloured, chlorophyllless flower petals of *P. hybrida* are able to light energy storage that can be directly detected using photoacoustic spectrometry. The corresponding photosynthetic process (ADAPFP), as well as the usual chlorophyll photosynthesis, is accompanied by additional ATP synthesis. However, this process is chlorophyllless and anoxygenic, featuring the following characteristics, which differ from those of the usual chlorophyll photosynthesis in green plant tissues:

- (1) There is a positive PA signal shift in response to strong continuous light switching on at high (>150 Hz) frequencies of modulating light, whereas such a shift for green photosynthetic tissues is negative. This is most likely because the proposed ADAPFP is more efficient under high light intensities than under moderate or low light intensities, in contrast to usual chlorophyll photosynthesis, which reaches saturation and photoinhibition under strong light intensities.

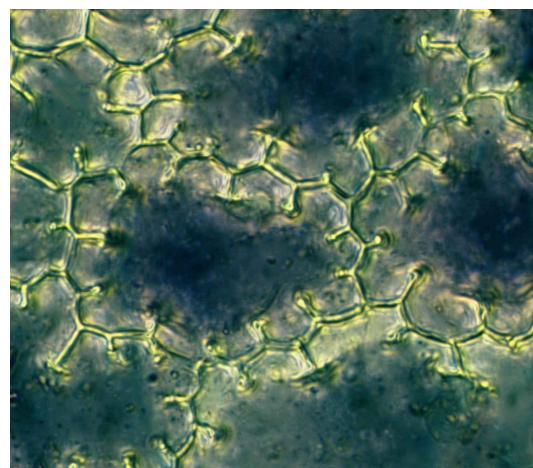


Figure 4 | Adaxial flower petal epidermis of *P. hybrida*. Bottom view. The image is created by combining the total of 8 images focused at different depths of the field. It can be seen that ingrowths of the epidermal cell wall are terminated by a widenings closely adjoining the vacuole. See also the 3D-model (Supplementary Data 2).

- (2) There is a long lag period under strong light, lasting a few minutes, while the usual chlorophyll photosynthesis requires 30–40 s to be fully operable under moderate light. Consequently, the proposed chlorophyllless photosynthesis is difficult to detect when, as is conventional in the case of chlorophyll photosynthesis, its PA signal is measured in response to strong continuous light after dark adaptation. In contrast, the PA-signal of the ADAPFP must be detected in response to a dark continuous test pulse, interrupting the strong continuous background light.
- (3) The ADAPFP is not dependent on chlorophyll but is performed only in the presence of anthocyanins, which are located mainly in the flower epidermal vacuoles.
- (4) In contrast with the usual chlorophyll photosynthesis, ADAPFP cannot be excited by blue light, at least in *P. hybrida*.

Special efforts should be made in future studies to clarify the potential relationships between the discussed anthocyanin-dependent (and possibly membrane-related) photochemical processes and ingrowths of the epidermal cell walls as far as the membranes of the anthocyanin-containing vacuoles were (Fig. 4) connected to these ingrowths. Nevertheless their simple rigidity-supporting function that is necessary for strengthening of a conical (well known) shape of epidermal petal cells cannot be excluded.

The molecular mechanisms of the proposed chlorophyllless ADAPFP are absolutely unknown, but they are not impossible in terms of the known photochemistry of anthocyanins. The conventional conception of the photosynthetic process requires the existence of a chromophore molecule that is capable of light excitation to singlet states. This molecule is chlorophyll in plants. However among the different basic forms of natural anthocyanins that can exist in aqueous solutions between pH 1–8, interconverting via pH-dependent equilibria, only the 7-hydroxyflavylium ion (AH⁺) can be excited by the quanta of light energy to its singlet counterparts (AH⁺*)¹⁹. A very fast (6–20 ps) proton transfer from AH⁺* to water is followed by fast (ca. 200 ps) decay of the resultant excited base (A*) back to the ground state (A). In addition, A is attacked by the nucleophilic water molecules, resulting in the formation of hemiacetal (B) at pH > 3¹⁹. From this knowledge, we might formulate a preliminary working hypothesis that there is a vacuolar membrane pool of A equilibrating with its free forms: A or AH in vacuole and/or B in cytoplasm. After A captures a proton from the cytoplasm and undergoes light excitation, it ejects the captured proton to the vacuole but



Figure 5 | Blue, white and red corollas of *P. hybrida* 'Dreams Appleblossom' plants. Photograph by V. Lysenko.

not to the cytoplasm. Note here that the quinonoidal base *A* is blue at pH 6.0–7.0²⁰, corresponding to the approximately neutral pH of the vacuoles of blue petunia flower petals²¹. Hemiacetal *B* is colourless or pale yellow with a weak-acidic to neutral pH (at least >3)¹⁹, corresponding to the neutral pH of the cytoplasmic environment.

Such a light-driven proton pump could possibly operate simultaneously with vacuolar membrane ATPase (V-ATPase)²², creating a proton gradient (Δ pH) across the membrane to produce acidic vacuolar sap. In this context, Δ pH is of interest as a potential source of energy driving a hypothetical ATP-synthase, similarly to that occurring in thylakoids of green plants. This mechanism (if it exists), however, would require an exact control of the forward and back transmembrane fluxes of protons to support a neutral pH in the cytoplasm, which is strictly necessary for most of the cytoplasmic enzymes. The independence from any redox electron transport chains, as well as the quick deactivation of excited singlet state of anthocyanins (*A*^{*}) to the ground state (*A*), is another specific characteristic of the proposed Δ pH “pumping” mechanism, making it unable to generate ROS, and, therefore, to be safe and operable under high light intensities. The latter is in accordance with the discussed above results of our photoacoustic studies.

However, it is unclear whether the AH^+ could exist in a membrane-bound form, considering that AH^+ molecule (red) is stable only at acidic pH values: thus, at pH 4.5, the equilibrium molar fractions of *A* and AH^+ are approximately equal²³. Moreover, anthocyanins are water soluble pigments. Therefore, possible complexes between flavylium derivatives and other membrane and vacuolar components, including proteins, must be considered to assume the stability of their proposed membrane-bound forms. It is interesting in this context that natural anthocyanins penetrate the outer part of the external lipid layer of liposomes that are composed of erythrocyte lipids²⁴. Evidently, just membrane-bound anthocyanins would be of special interest from the point of view of their photochemical behaviour.

Nevertheless, the redox chain mechanism cannot be excluded as an alternative way to explain the ADAPFP. Thus, natural anthocyanins²⁵ and their synthetic analogues (flavylium salts)²⁶ were employed as sensitizers for dye-sensitized solar cells (DSSCs). Dyes were absorbed into a porous TiO_2 layer (photoanode) that extended the semiconductor's performance allowing it to collect photons at a lower energy level. Following light irradiation, the dyes were excited to state *A*^{*}, performing the subsequent electron injection into the photoanode ($A^* + TiO_2 \rightarrow A^+ + (TiO_2)^-$). Therefore, the anthocyanin-dependent utilisation of light energy in natural redox chains also seems possible, but it is clear in this case that the H_2O molecule is not a primary electron donor as in oxygenic photosynthesis.

An example of chlorophyllless photosynthesis, based on the redox chain mechanism, was recently shown to be dependent on light absorption by the green fluorescent protein (GFP) in jellyfish²⁷. The authors demonstrated the photoinduced electron transfer from GFP to tetrazolium, quinone, FMN^+ and NAD^+ . A carotenoid-

dependent, chlorophyllless photosynthesis was shown in aphids²⁸. The carotenoid-containing aphids with an orange phenotype (in contrast to a greenish phenotype) exhibited a light-dependent increase in the $NADH/NAD^+$ ratio and ATP content, and their extracts were also able to perform the photoinduced reduction of tetrazolium. In this connection, it was proposed that the photosynthetically reduced NADH was being utilised as an external electron donor by the mitochondria for ATP synthesis using known pathways. Both of these proposed types of photosyntheses, GFP-based and carotenoid-based, can be reliably interpreted in terms of redox chain mechanisms as the photosynthetically synthesised reducing equivalents originate from the cytoplasm and are easily transported to the mitochondria.

Assuming the redox interpretation of ADAPFP, it is necessary to propose that the reducing equivalents are synthesised in the vacuole and, therefore, that they would hardly be transferred to the mitochondria across the vacuolar membrane. Another potential explanation could be based on the anthocyanin-driven light excitation of some unknown redox chain in the vacuolar membrane coupled with a proton pump, similar to the thylakoid electron transport chain. However, the existence of such a hypothetical vacuolar membrane chain is not supported by any evidence. Therefore, the proposed non-redox proton pump mechanism seems to be a little more preferred because it does not require the assumption of any additional unknown metabolic pathways and is implied with a simple and known reaction of proton transfer from the excited 7-hydroxyflavylium ion to water.

The discussed here hypothetical mechanisms of ADAPFP allow for diverse interpretations considering the obtained biophysical and biochemical data. This has an objective cause as far as the light reactions studied in this work are involved in photosynthetic processes, which, for the first time, were shown to be located in the vacuoles. In summary, the basic results of our work showing the existence of ADAPFP remain a wide field for explanations as to how light energy storage could lead to ATP synthesis in anthocyanin-containing flower petals. We hope that the uncertainty and difficulties in the interpretation of the molecular mechanisms of these processes will stimulate further research in this area.

Methods

Plant material. *Petunia hybrida* 'Dreams Appleblossom' plants with blue, white and red corollas (Fig. 5) were cultivated to floral maturity in a greenhouse on a vermiculite-perlite-peat (2 : 1 : 2) mixture under standard conditions (temperature $25 \pm 1^\circ C$; relative air humidity 70%; relative soil moisture 60%; light/dark regime 16 h/8 h; full sun PAR – 1800 μmol photons $m^{-2} s^{-2}$ for 6 h and shade light – 300–500 μmol photons $m^{-2} s^{-2}$ for 10 h). Fully opened corollas, 5–6 cm in diameter, were collected shortly before anthesis.

In preliminary experiments, the residual chlorophyll (*a* + *b*) content in *P. hybrida* was found to be 19 ± 4 $\mu g/gDW$ for flower petals (peripheral part of the corollas; both white and blue) and 2100 ± 100 $\mu g/gDW$ for leaves. The chlorophyll determination was performed spectrophotometrically, according to Lichtenthaler and Buschmann²⁹, using the chloroplast isolation method of Weiss et al.¹ as an intermediate step to avoid any interference of anthocyanins. The determination of



chlorophyll content in white petals was also performed using direct chlorophyll extraction from petal tissues to confirm the results obtained during the chloroplast isolation step. In this case, the chlorophyll content was $18 \pm 2 \mu\text{g}/\text{gDW}$.

Adjusting photon fluxes. The photon fluxes were adjusted to necessary levels using a spectroradiometre MS-12 (Belarusian State University, Belarus).

Photoacoustic studies. The evaluation of light energy storage (ES) was performed in accordance with the generally accepted principles and practical approaches^{5–7,30} using the previously described fast Fourier transform (FFT) photoacoustic method¹³ with modifications. Leaf discs (10 mm in diameter) were cut from the peripheral part of the corollas where the residual chlorophyll content is minimal. The disks were placed in the photoacoustic chamber (glass, 200 μL) communicating with an electret microphone.

Weak measuring light emitted from white LED was modulated by an optical chopper at 40 Hz or 280 Hz (light duty factor 50%) and at a 1-ms pulses with a period of 25 ms to obtain the FFT PA signal. Strong non-modulated background light was emitted from powerful white 20 Wt LED to achieve an instantaneous intensity of 36 or 900 photons $\text{m}^{-2} \text{s}^{-1}$. A bifurcated fibre-optic cable conducted the light beams from modulated and background sources to the measuring photoacoustic cell. An identical reference cell was used to help cancel the background noise from the surroundings. The microphone signals from both of the cells were passed through a low-noise differential amplifier subtracting them from each other, and then the output signal was passed to the PC sound card. Real-time signal data processing was carried out to perform FFT using the SpectraLab program package. The following settings were applied: FFT size, 131,072 samples; sampling rate, 192,000 Hz; sampling format, 16 bit; and spectral line resolution, 1.47 Hz.

The real-time FFT-peak amplitude of a PA signal at a specified frequency (PA_{RTA} ; Supplementary Fig. S1) was measured in 0.5-s intervals and the obtained values were saved to a log file (*.xls). The logged data were processed to obtain time-resolved PA signal transients (Fig. 1) using standard tools of the Excel and Origin program packages.

The ES was calculated as $(\text{PA}_{\text{max}} - \text{PA}_{\text{min}})/\text{PA}_{\text{max}}$, where PA_{max} and PA_{min} are the maximal and minimal PA amplitude values, respectively, within the PA-transients measured immediately before and after (or in a reverse order) switching the background light on/off (see Fig. 1).

Dry blue paper was used as a standard sample to check the linearity of the acoustic signal and signal processing system (Fig. 1f, curve 3). In this case, the ES did not exceed the noise level (0.6%), showing that PA signals measured at low light intensity (36 photons $\text{m}^{-2} \text{s}^{-1}$; modulating light) and at high light intensity (936 photons $\text{m}^{-2} \text{s}^{-1}$; continuous background + modulating light) are equal and, therefore, that the signal is linear.

In separate experimental series, PA signals were excited by short and strong light pulses (0.15 ms, white LED, 900 μmol photons $\text{m}^{-2} \text{s}^{-1}$, period 10 ms). The obtained individual PA-pulses (spikes) are shown in Supplementary Fig. S2b. Each spike of interest was selected from the common *.avi file and saved as text data in an *.xls file using SpectraLab program. The shape and time delay of the differential PA signal components (“differential spike”) that were generated in response to the continuous dark period (related to energy storage) were calculated by averaging 100 individual PA spikes that were detected during 1 s before and 1 s after strong continuous light (white LED, 900 μmol photons $\text{m}^{-2} \text{s}^{-1}$) switching off (Supplementary Fig. S2a). To obtain the differential signal, both of these averaged signals were subtracted from each other. The time delay (retention time) of the amplitude maximum of such a differential PA spike was calculated from the beginning of the exciting light pulse (Fig. 2).

ATP and ADP determination. After 30 min of dark adaptation, petunia plants with white and blue corollas were illuminated (white LED, 900 μmol photons $\text{m}^{-2} \text{s}^{-1}$, 2700 K) during specified time intervals (30–180 s); their peripheral parts were detached shortly before (5–6 s) the end of these intervals and were rapidly frozen in liquid nitrogen. Blank experiments were performed with non-illuminated, dark adapted plants. The ATP and ADP levels in the frozen tissues were analysed using HPLC as described by Liu H. et al.³¹.

Determination of the O₂ levels in an airtight chamber in the presence of plant tissues. Flower petals or leaf samples (5 cm²) were placed in an glass airtight chamber 20 × 30 × 1 mm in size having two syringe pierces septa. The chamber was submerged in a temperature-controlled water bath at 25 °C to avoid light heating which, in this case did not exceed 0.5 °C. After 30 min of dark or light exposure (white LED, 900 μmol photons $\text{m}^{-2} \text{s}^{-1}$), 10 μL -gas probes were sampled from the chamber with the simultaneous injection of 2 M NaCl for pressure equalisation. The samples were analyzed as described by Carle³² using a gas chromatograph Tswet-560 (Tswet, Dzerjinsk, Russia) equipped with a thermal conductivity detector. The O₂ level was calculated as $iS_{\text{O}_2}/(iS_{\text{O}_2} + jS_{\text{N}_2})$ where S_{O_2} and S_{N_2} are the areas, respectively, of O₂ and N₂ chromatographic peaks, i and j are the volume response factors for O₂ and N₂ as determined using a calibration gas mixture. The preliminary experiments showed that “dark” and “light” O₂ levels, measured after 30 min of exposure in the chamber, are close to their equilibrium points in both the flower petals and green leaves. Thus, these levels measured at 20 min are usually are about 92–95% of the levels measured 30 min after the beginning of the exposure.

Light microscopy of flower petals tissues. The flower petal epidermis of P. hybrida was detached from the petals using a simple adhesive tape stripping technique. The

flower petals were placed between two pieces of adhesive tape, slightly pressed against each other and then peeled off from each other. The adaxial epidermal cell monolayer remained adhered to one of these pieces. The obtained monolayer preparations were viewed from the backside of the epidermis using a light microscope equipped with a 60× plan apochromat objective and a CCD camera. A total of 8 images were made at different depths of focus. The focused areas were combined to create a single completely focused image and 3D-model using the HeliconFocus software. The 3D model was inverted along the y-axis to simulate a top view.

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Author contributions

V.L. conceived the project, performed the photoacoustic and gas chromatography studies and wrote the paper. T.V. performed HPLC determination of ATP and ADP. V.L. and T.V. contributed in light microscopy studies and image data processing.

Additional information

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