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Feature Review

Light Emission in Betalains: From Fluorescent Flowers to Biotechnological Applications

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The discovery of visible fluorescence in the plant pigments betalains revealed the existence of fluorescent patterns in flowers of plants of the order Caryophyllales, where betalains substitute anthocyanins. The serendipitous initial discovery led to a systemized characterization of the role of different substructures on the photophysical phenomenon. Strong fluorescence is general to all members of the family of betaxanthins linked to the structural property that the betalamic acid moiety is connected to an amine group. This property has led to bioinspired tailor-made probes and to the development of novel biotechnological applications in screening techniques or microscopy labeling. Here, we comprehensively review the photophysics, photochemistry, and photobiology of betalain fluorescence and describe all current applications.

Highlights

The discovery of betalains' fluorescence property opened up new fields in the research and applications of the pigments.

Similarities in fluorescence characteristics point to betalamic acid as the active compound.

Fluorescence allows visualization and staining of biomolecules, cells, tissues, and parasites.

Novel microscopy, biosensing, and high-throughput techniques have been developed based on the fluorescence of natural or tailor-made betalains.

Betalains

Betalains are nitrogenous plant pigments that are characteristic for plants belonging to the order Caryophyllales. These pigments are divided into the yellow betaxanthins and the violet betacyanins [1]. The presence of both types of pigments is required for the orange and red colors that coexist in nature with the pure yellow and violet colors. Betalains substitute anthocyanins and play their roles in the colored tissues of most plant families of the Caryophyllales. Both families of pigments are water-soluble and mutually exclusive [2,3]. Numerous studies were performed on the color properties of betalains since they were described as a novel family of pigments, different to 'nitrogenous anthocyanins' [4,5]. However, it was not until 2005 that their natural fluorescence was discovered [6]. In addition, it was demonstrated that under physiological conditions, light emission was exhibited in the plant tissues that contain them, including flowers [7,8].

Among the Caryophyllales plants, red beet roots (*Beta vulgaris*) and the fruits of cacti belonging to the genus *Opuntia* are the best known sources of betalains, with betanin and indicaxanthin being, respectively, their main pigments [9,10]. Current research has described the betalain content of novel sources, such as the tubers from *Ullucus tuberosus* [11] or the betalain-containing berries of *Rivina humilis* [12]. Also, the multiple shades of quinoa grains (*Chenopodium quinoa*) have recently been reported to be based on betacyanins and betaxanthins [13]. In addition, betalains have in recent years been shown to have promising bioactive properties. Early investigations revealed a strong free radical scavenging capacity of betalains purified from beet root [14]. Subsequent research revealed the existence of an intrinsic activity, present in all betalains, that is modulated by structural factors [15,16]. Furthermore, studies with different human cancer cell lines have demonstrated the potential of betalains in the chemoprevention of cancer [17,18]. *In vivo* experiments have shown that very low concentrations of dietary pigments inhibit the formation of tumors in mice [19,20] and extend the lifespan of the model animal *Caenorhabditis elegans* [21]. The bioactivities described are supported by the high antiradical capacity of the pigment's structural unit, betalamic acid, and point to a promising potential of betalains in tumor prevention *in vivo* and a possible role for betalains in a health-promoting diet [22]. High bioactive potential and a strong fluorescence, both maintained under mild, physiological conditions, are fueling current research on betalains, which is enjoying a golden age [23].

The discovery of betalains' fluorescence opened up new fields in the research and applications of these pigments, outstripping initial expectations when the phenomenon was first discovered [6]. Betalains' fluorescence is now used for a wealth of applications, ranging from the detection of malaria-infected erythrocytes to the screening of enzyme activities for opioid-forming microbial factories.

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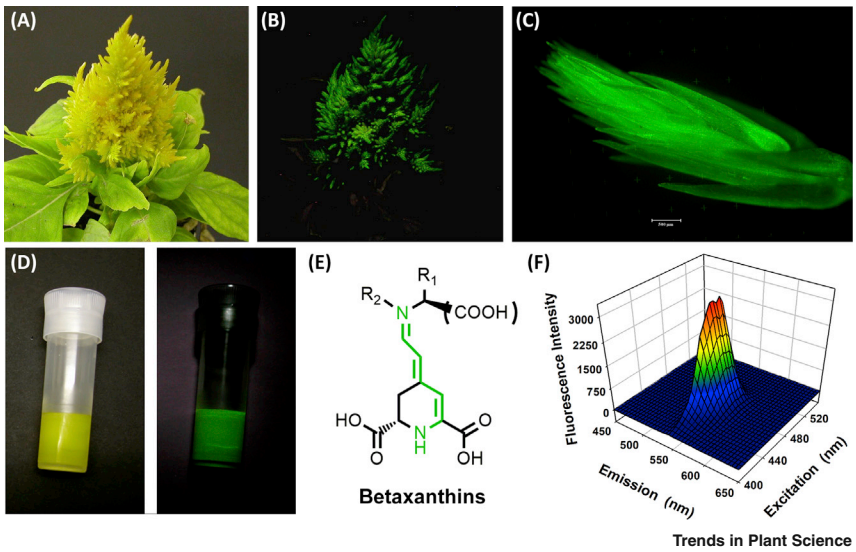

Figure 1. Fluorescence of the Plant Pigments Betalains.

(A) Macroscopic image of the inflorescences of a yellow feather cockscomb, *Celosia argentea* var. *plumosa*, under white light. (B) The same yellow specimen visualized by fluorescence under blue light stimulation. (C) Close view of inflorescence under blue light in a Leica DMRB microscope with incident light beam, using the filter cube I3 (excitation: 450–490 nm). (D) Tube containing a frozen solution of pure dopaxanthin under white light (left) and blue light stimulation (right). (E) General structures for betaxanthins indicating the electron resonance system responsible for fluorescence. (F) 3D-fluorescence spectrum for the betaxanthin dopaxanthin in a 3 μM solution in water at 25°C.

Here, we review recent insights on the fluorescence of betalains, including a description of the initial discovery, basic and applied perspectives, and possible future trends.

Initial Discovery of Fluorescence

The discovery of fluorescence in betalains was linked to research on their biosynthetic pathway. The steps of the biosynthesis of betalains were preliminarily established in the late 1960s and two main enzymes were proposed to build the structural units of the pigments. A 4,5-DOPA-extradiol-dioxygenase (DODA) was proposed to cleave the aromatic ring of L-dihydroxyphenylalanine (L-DOPA) to a linear form, able to produce the basic structural unit of all betalains, betalamic acid [24,25]. L-DOPA is an important precursor to form betalamic acid, but also the precursor molecule of the additional substructure present in violet betacyanins, the *cyclo*-DOPA moiety. Tyrosine hydroxylation to L-DOPA by tyrosinase and further oxidation by the same enzyme to promote cyclization to *cyclo*-DOPA were considered to be the additional steps to form betaxanthins and betacyanins. Experiments performed mainly with radioactive labeled precursors configured a simple biosynthetic pathway that was reviewed in the early 1980s in a work that established the accepted route at the time [26]. The derived scheme was considered definitive until inconsistencies in the proposed reactions with the enzymatic-chemical mechanism of tyrosinase-catalyzed reactions were pointed out by our group [1,27]. The new biosynthetic net considered betaxanthins not only as final products of a linear pathway but also as possible intermediates in the formation of other pigments. In this context, characterization of novel reactions in the biosynthetic pathway of betalains promoted the search for singular pigments in flowers of different plant species [27] and the development of novel methods for their semisynthesis and purification [28]. As a result of those protocols, pure hydroxylated betalains were obtained to be used as substrates of the key enzymes. The resulting diluted pigment solutions needed to be concentrated and, due to the strongly hydrophilic nature of betalains, the technique used was freeze-drying. Upon freezing in liquid nitrogen, diluted aqueous solutions of betaxanthins exhibited a strong fluorescent glow, which was visible to the naked eye (Figure 1D). Freezing a fluorescent solution causes an

increase in the light emitted due to an increase in the quantum yield of fluorescence. This happens when molecules slow down their vibration and energy loss from excited states finds a preferential relaxation through fluorescence. This serendipitous discovery provided the first evidence for the existence of fluorescence in betaxanthins' solutions [6–8]. The previous 50 years of research on the betalain pigments [5,29] had not considered fluorescence.

After the initial surprise and awareness of the possible relevance of fluorescence in pigments naturally present in flowers, strong efforts were made to ascertain pigment purity and to characterize multiple structures, of both betaxanthins and betacyanins, and to analyze plant extracts from different sources [7,16,28]. In addition, a photographic filter system was used to visualize fluorescence and suppress the contribution of reflected light. Filters used for fluorescence photography were specially designed to enable green fluorescence detection, while suppressing UV excitation or emission [7]. Microscopic visualization of betaxanthins in plant tissues was technically easier because the recorded fluorescence spectra indicated a similar behavior to that obtained for the broadly used GFP system, previously captured with standard microscopy filter cubes. *Mirabilis jalapa*, *Portulaca grandiflora*, *Lampranthus productus*, and *Carpobrotus acinaciformis* were described in the initial publications [6–8] but many other single molecules [30] and plant extracts were analyzed to support the novel phenomenon. This is the case for *Celosia argentea*, whose yellow inflorescences appear to glow under the blue light stimulation system developed, as shown in Figure 1A,B. In this case, the fluorescence detected in the yellow flowers is mainly due to the presence of the pigments vulgaxanthin I and miraxanthin V. In all cases, the use of epifluorescence microscopes allows the structure of the flowers to be seen in detail (Figure 1C).

Eventually, the biosynthetic pathway in plants turned out to be more complex than expected, with multiple possible branches in the scheme and with a new family of cytochrome P450 enzymes resolving the inconsistencies detected for tyrosinase [1,31]. This was another surprise in recent betalain research, which relegated tyrosinase to a secondary role after promoting research in the biosynthetic pathway of the pigments and the discovery of fluorescence.

Structure–Activity Relationships

Systematic work has been performed with multiple betalains under the same conditions and has enabled the characterization of substructures that enhance or weaken the fluorescence described for the pigment's family. In this sense, it was initially reported that when electron density is withdrawn from the resonating system higher fluorescence can be expected. This is exemplified by the high fluorescence exhibited in the pigment miraxanthin I, derived from methionine sulfoxide when compared with that corresponding to methionine-betaxanthin [30]. The betaxanthins vulgaxanthin I and vulgaxanthin II [32], derived from the amino acids glutamine and glutamic acid, respectively, present the same situation and the glutamic acid-derived pigment presents higher fluorescence intensity due to the withdrawing effect of the carboxyl group. A similar effect was observed for semisynthetic betalamic coumarins [33]. When an electron-donating methyl group was substituted from cBeet120 by an electron-withdrawing trifluoromethyl group at the C4 position of the coumarin moiety in cBeet151, the molecule became more fluorescent, with quantum yields of 0.021. The effect of electron density-donating groups in betalain fluorescence is the opposite and fluorescence intensity is reduced by the presence of hydroxyl groups [16]. The presence of the extra carboxylic group in dopaxanthin and tyrosine-betaxanthin (also called portulacaxanthin II) strengthens the fluorescence with respect to dopamine- and tyramine-betaxanthin [30]. In addition, other structural considerations should be taken into account and the effect of groups able to generate hydrogen bonds has been explored [34]. The existence of intramolecular hydrogen bonds may favor structurally constrained structures, as occurs in miraxanthin I. This reduces radiationless losses of energy and explains the high fluorescence quantum yield of this molecule (Table 1), described as the most fluorescent betaxanthin since the early description of the phenomenon [30].

The study of structure–activity relationships in betalain fluorescence is also related to the effect of substructures on the absorbance of the molecules. This way, the effect of carboxylic acid groups

Table 1. Quantum Yield of Fluorescence Obtained for Studied Betalains

Betalains	Φ_F in water	Φ_F in methanol	Φ_F in ethylene glycol	Refs
Betanin	0.0007	0.0013	0.0047	[37]
Indicaxanthin	0.0053	0.0081	0.033	[39]
Miraxanthin V	0.003	0.0047	0.015	[41]
Vulgaxanthin I	0.0073	0.011	0.039	[40]
Miraxanthin I	0.0084	–	–	[34]

on the fluorescence of betalains supported existing information on the effect of this group on absorbance spectral properties. The betanin spectrum displays a maximum at 536 nm, but a hypsochromic shift occurs with the decarboxylation of the structure at C-2 [35]. A greater shift, in the same direction, occurs in the 14,15-dehydrobetanin compound (neobetanin), which is orange and not violet [36]. The same effect is observed in betaxanthin absorbance, and a hypsochromic shift occurs with the decarboxylation of tyrosine-betaxanthin and dopaxanthin.

A linear relation can be found in the decrease of the Stokes shift (separation between excitation and emission spectra in fluorescence) with increasing excitation wavelengths [7]. In contrast to the highly fluorescent betaxanthins, their violet counterparts, betacyanins, are only weakly fluorescent. Fluorescence in this case is mainly detected in the presence of carboxylic groups and in the absence of hydroxyl ones. This enhances the fluorescence of the molecule, as occurs in the case of the indoline-derived betacyanin [16]. Betanidin and the glucosylated pigment betanin are only weakly fluorescent, with a very short excited-state lifetime, determined for betanin as 6.4 ps in water [37]. Maximum excitation wavelengths in betacyanins occur between 521 and 529 nm and emission spectra are centered around 570–575 nm.

Nowadays, a wide variety of synthetic and natural betaxanthins have been obtained and their fluorescent properties have been characterized. All the pigments show similar behavior, with excitation maxima between 471 and 474 nm (blue color) and emission maxima between 548 and 551 nm (green color) [16]. Similarities in fluorescence characteristics point to the responsibility of betalamic acid in fluorescence (Figure 1E,F). The nature of the amine or amino acid moiety has a limited contribution to the final spectral characteristics and there are no significant differences in relation to the chain length or its polarity.

Photophysics

When betalains are photoexcited, they undergo an excitation of their molecules, causing them to pass from the initial resting state S_0 to the excited state S_1, according to Jablonski's diagram (Figure 2). This corresponds to the absorption measure obtained by means of UV-visible (UV-vis) spectroscopy, where a maximum absorption peak appears around λ_{max} = 475 nm for betaxanthins and λ_{max} = 536 nm for betacyanins [16].

The study of the emitted fluorescence has shown that this $\lambda_{max\ abs}$ corresponds to the λ of excitation which produces the maximum fluorescence emission around λ = 550 nm for betaxanthins and λ = 575 nm for betacyanins. The subsequent relaxation of these molecules is carried out largely by internal conversion. This process releases the acquired energy in the form of heat. This energy is dissipated by convection of the excited molecules of the chromophore towards the adjacent molecules of the solvent. Only a small part of it is released as fluorescence. The quantum yield for fluorescence (Φ_F) gives low values if compared with compounds like rhodamine 6G, which has a value Φ_F = 0.95 [38]. In betalains it has been demonstrated that this value is proportional to the viscosity of the solvent used (Table 1). Thus, the analysis in water and methanol gives lower values than in ethylene glycol [37,39–41]. This response has also been shown for betaxanthin molecules present in gelificating media, where fluorescence intensity increases with the time course of the process [42]. These results

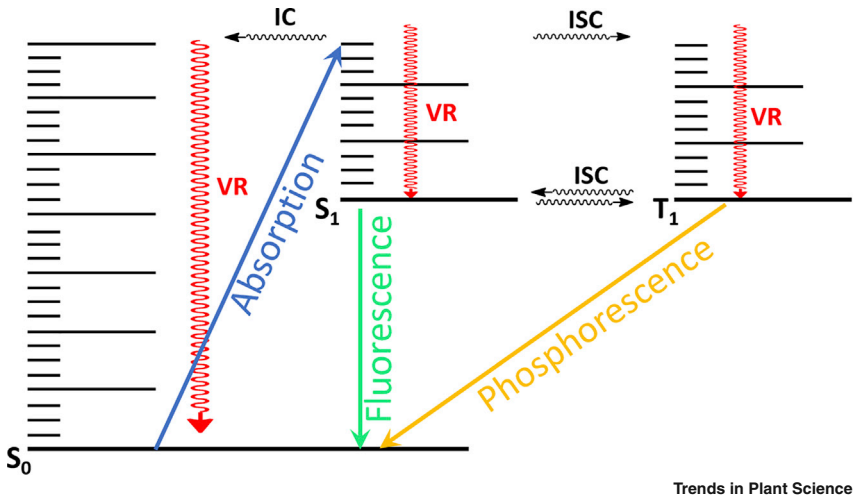

Figure 2. Jablonski's Diagram for the Excitation and Relaxation of Absorbing Molecules.
Betalains, after photoexcitation (absorption), experience relaxation by internal conversion (IC) or by light emission (fluorescence). Triplet T_1 state (phosphorescence) has been described for betalains after the addition of potassium iodide (heavy-atom effect) and triplet sensitizers [37,39–41]. IC decreases in viscous media, thus increasing the quantum yield of fluorescence. Abbreviations: ISC, intersystem crossing; S_0, resting state; VR, vibrational relaxation.

show that an increase in the viscosity of the medium makes the convection of the acquired energy difficult, favoring the path of the fluorescence. The presence of aromatic rings linked to the electron resonance system characteristic of betalamic acid implies a marked decrease in the fluorescence intensity. Only the fully planar aniline-derived betalain presents fluorescence intensity comparable with betaxanthins. The effect of the medium was also studied by taking indicaxanthin, vulgaxanthin I, and miraxanthin V as model betaxanthins, describing how higher viscosity in the solutions increased the fluorescence quantum yields and the lifetimes of the excited states [39–41].

Transient absorption spectroscopy UV-vis-near infrared has been used for the characterization of the properties of the S_1 state, since this excited state has a very short life [37,39–41]. The excitation wavelength is adjusted to the electronic transition $S_0 \rightarrow S_1$ and is close to the maximum of the stationary absorption. The graph resulting from this analysis (Figure 3) shows positive and negative bands. The positive bands correspond to the transitions $S_1 \rightarrow S_n$ ($n > 1$),while the negative bands correspond to the bleaching of the S_0 state and to the stimulated emission $S_1 \rightarrow S_0$. In this sense, the existence of conical intersections between the S_0 and S_1 states has been demonstrated [43]. This finding is related to torsional geometries of the molecules and could partially explain the quantum yields of fluorescence determined for betalains and the effect of solvents.

Over time (ps scale), it is observed that a triplet T_1 state, by intersystem crossing, is not produced from state S_1, but the complete recovery of the S_0 state occurs, with the consequent deactivation of the excited molecules. The absence of the T_1 state prevents the formation of toxic oxygen species by energy transfer, which supports the theory of the protective character of betalains in plants containing them. In extracts of vulgaxanthin, $Ru(bpy)_3^{2+}$ was used as a triplet sensitizer [40]. Nanosecond photolysis at λ_{exc} = 355 nm of the mixture $Ru(bpy)_3^{2+}$ with vulgaxanthin leads to the initial absorption band at λ_{max} = 360 nm. Its decay is accompanied by the appearance of a new positive absorption band with a maximum at λ_{max} = 540 nm, which can be assigned to vulgaxanthin in the T_1 state. At 2500-ns delay, as well as the positive band, there is also a negative band at 460 nm, mainly reflecting vulgaxanthin ground state bleaching [40].

One appropriate way of achieving an increase in the lifetime of the S_1 state is by increasing the viscosity of the solvent, which seems to indicate a significant change in the geometry of the molecules

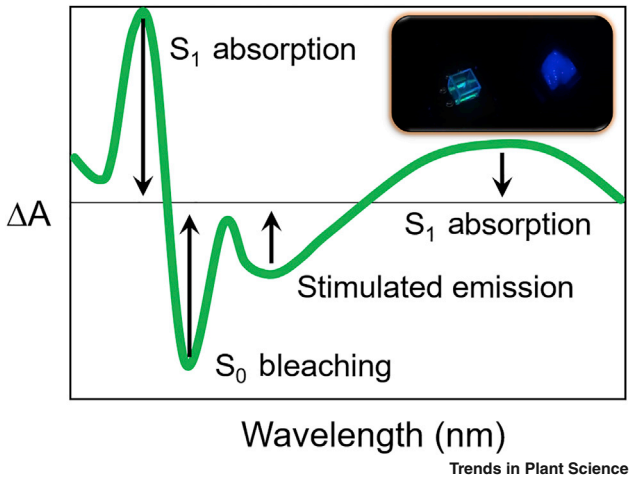

Figure 3. General Scheme for the Transient Absorption Spectra UV-Visible-Near Infrared of Betalains. Positive bands correspond to transitions from the excited (S_1) state and negative bands correspond to the bleaching of the resting (S_0) state. Inset: green fluorescence in a 3 μM solution of miraxanthin I coming out from a 3 ml quartz cuvette placed in a Shimadzu RF-6000 spectrofluorometer (excitation at 470 nm).

that precedes the $S_1 \rightarrow S_0$ radiationless transition involved in the deactivation of S_1 state. In betanin, molecular rotation around the $C_{12} = C_{13}$ bond may accelerate the internal conversion process [37]. The increase in the viscosity of the medium causes the lifetime of the S_1 state to pass from 7.7 ps in methanol to 27 ps in ethylene glycol. This higher viscosity of the medium hinders the molecular rotation, which in turn decreases the energy dissipation by convection and favors the emission of fluorescence; this is consistent with the results obtained in the quantum yield of the fluorescence.

By means of NMR it has been shown that betalains present a mixture of E/Z stereoisomers. This phenomenon has been corroborated with transient spectroscopy. Two different time constants appear, corresponding to the excitation of each stereoisomer to its excited state, which subsequently relaxes, presenting a deactivation spectrum in both cases, similar to Figure 3. These deactivation spectra of the S_1 state are the fingerprint of betalains and are also observed in aqueous extracts of plants of the order *Caryophyllales*. Extracts of *Opuntia ficus-indica*, where indicaxanthin is the major pigment, show a transient absorption spectrum at $\lambda_{exc} = 483$ nm, similar to that obtained in pure indicaxanthin aqueous solutions [39], whereas extracts of *Phytolacca americana* berries show photophysical characteristics similar to extracts of pure betanin [37].

Biological Relevance of Fluorescence

Color acts in plants as a signal for communication with other species. In general, plants take advantage of visual signals to attract animals' attention for the purpose of pollination and seed dispersal. In flowers, color represents an important characteristic known to attract pollinators [44–46]. The capacity of insects to detect symmetry and asymmetry, and the preferences described for special patterns [47], confer relevance to color modulation in flowers and, therefore, to the optical properties of the underlying pigments. The establishment of the fluorescent properties of individual pigments and flower extracts raised the question whether flowers containing betaxanthins could be considered as fluorescent items.

To observe the fluorescent phenomenon, the incident light was filtered in order to avoid contamination of the emitted fluorescence. Based on the properties of betaxanthins in aqueous solution, a photography filter system especially designed for green fluorescence visualization was used [7]. The resulting images showed how fluorescence is maintained in the physiological environment, as shown in Figure 1B. Betacyanins are only weakly fluorescent [16] and, due to the overlapping

observed between the betacyanin absorbance and the betaxanthin emission spectra, natural betacyanins are able to absorb a high degree of the light emitted by betaxanthins [8]. This was described as an unedited inner filter effect that was able to generate contrasting patterns in the flower fluorescence signal.

The relevance of light emission in flowers for the attraction of pollinators is a matter of current debate [48–52]. The weakness of the signal in comparison with color absorption or light reflection may limit the role of fluorescence as a stand-alone communication signal. However, fluorescence may support other well-established signals and not necessarily substitute them. Some species of spiders display distinctive fluorescent signals depending on gender and life stage, with females maintaining the brightness throughout their lives [53]. Fluorescent external areas may serve as an additional attracting signal for preys or males. In any case, some interactions that rely on emitted fluorescence have already been described in nature, such as those involved in mating budgerigars [54,55], or in attracting prey to some species of jellyfish [56]. In the latter case, fluorescence is derived from a GFP with analogous fluorescence spectra to betaxanthins, which appears in yellow patches in the tentacles. Remarkably these patches are close to nonfluorescent purple ones, described by the authors to increase the contrast of the fluorescent region [56]. Green fluorescence and purple contrasting patterns constitute the same tandem described for betaxanthins and betacyanins in flowers and hypothesized to be of relevance in pollination [8].

It should also be considered that the different perception of light and the existence of receptors for specific wavelengths in the eyes of pollinators can make light emitted a major component of the perceived signal. This was the case hypothesized for betalains in cacti, since cactus-pollinating bats with specific receptors were reported as being able to see at the specific wavelengths emitted by betaxanthin fluorescence [8,57]. This hypothesis was confirmed by recent investigations that found how other species of bats are attracted from distances of up to 23 m by green light with a wavelength of $\lambda = 520$ nm [58].

Since betaxanthins are molecules that exhibit natural fluorescence in the visible range of the electromagnetic spectrum and this phenomenon is general to all betaxanthins, potentially any solution or structure containing them should be a glowing object. In this sense, the possible influence of fluorescence in seed dispersal has a major biological relevance. To date, only quinoa grains have been shown to contain significant quantities of betaxanthins in the yellow and orange varieties [13]. Color and fluorescence measurements in the grains corresponded to the colored outer layer of the grains, as visible under normal physiological conditions. Several identified betaxanthins give yellow coloration to quinoa grains and make them glow as intact viable grains. Thus, the discussion about the influence of fluorescence in pollination opens up to include a possible biological effect of this signal in seed dispersal.

Photostability of Betalains

Among the properties of betalains, their stability and color permanence have received great attention by researchers due to their applications in food coloring. Therefore, factors affecting stability during processing and storage, such as pH value, temperature, oxygen, and light, have been extensively studied since their early use by the food industry [59–62].

Exposure to light is one of the main factors affecting the stability of betalains, similar to other naturally occurring pigments such as anthocyanins or carotenoids. In addition, the bioactive properties attributed to these compounds, such as their antioxidant activity and free radical scavenging capacity, can be altered under light exposure [63]. Betalains obtained from several sources have been analyzed under dark and light conditions, such as the model betacyanin betanin extracted from *B. vulgaris* [64]. Pigment tolerance to light was found to be dependent on pH, and betanin in acidic solutions analogous to those found in plant cell vacuoles was stable, with a limited degradation due to light exposure [64]. In the case of the betaxanthin indicaxanthin [65], the presence of light increased its degradation over a wide range of pH values. Data were analyzed and adjusted to first order kinetics, with results

indicating a higher stability at neutral pH, in both the presence and absence of light in short time experiments. However, when indicaxanthin was exposed to light over long periods of time, the stability of the pigment considerably diminished at all pH values. The encapsulation of pigments in polymeric matrixes greatly improves their stability in long-term experiments. In this way the color of indicaxanthin became lighter after 6 months in the presence of light, which was linked to the destruction of the pigment. However, the color parameters did not change noticeably after storage for the same period of time in the absence of light [65]. In the same manner, the betaxanthin miraxanthin V and the betacyanin betanidin, the pigments with the strongest antiradical capacity, were affected by light exposure in solution over a wide range of pH [63]. Betanidin is known for being a very labile molecule that degrades quickly under working and storage conditions [59,66–68]. Although exposure to light reduced pigment stability, 50% of the initial pigment amount remained when encapsulated [63].

Stability of model betalains can be compared with that of their partner pigments anthocyanins. When anthocyanins' stability was analyzed after encapsulation in similar conditions to those used for betalains, it was observed that storage in the absence of light also reduced the pigment content [69,70]. The stability of anthocyanins was lower than that shown for miraxanthin V and betanidin and the presence of light accelerated anthocyanin degradation. Comparable results were obtained for a more divergent pigment, such as lycopene encapsulated in starch matrixes [71].

Biotechnological Applications of Fluorescence

Because betalain plant pigments are present in edible sources, the original discoverers of the fluorescence property suggested future applications for the food industry. Fueled by coetaneous interest in implementing fluorescence as an aesthetic color modulator [72], the idea arose to modify colors of food and beverages with fluorescent shades [6]. However, the real potential of betalain fluorescence was still to be discovered, thanks to contributions from groups all around the world (Figure 4). Precise fluorescent identification of pigments in natural sources led to methods for the visualization and staining of multiple structures and to biotechnological applications.

Improved Detection of Betalains in Plant Extracts

Fluorescence detection implies a high selectivity of the analysis, due to the specific excitation of the compounds. It has been extensively employed for the determination of different types of molecules in biosciences. Because few compounds emit a strong enough fluorescence for direct measurements, a derivatization procedure is normally necessary. This is not the case of betalains; the native fluorescence of the molecules can be directly applied to their detection. This implies an immediate improvement of standard detection protocols.

Betalains are usually found in complex mixtures in plant extracts and thus the most suitable means for identification and quantification involve high performance liquid chromatography (HPLC) separations. The direct use of fluorescence detectors after chromatographic separations for the accurate and sensitive quantification of betaxanthins was the first application of fluorescence. Improvement of detection involved the determination of traces of betaxanthins in white tissues of plants of the Caryophyllales [6]. Deeper analyses revealed an improvement in the limits of detection and quantification when compared with standard protocols based on absorbance [30]. The average reduction of the limits is around 76% of the previous values and also implies a reduction in the amount of sample needed in the assays. The wavelengths used in the detectors were 460 nm for excitation and 510 nm for emission, which were found to be suitable for detecting the native fluorescence of all the pigments assayed, independently of the elution time and of the percentage of organic phase of the gradients.

Fluorescent Probes in Microscopy

Fluorescence of betaxanthins has been used in microscopy applications to observe betaxanthin-containing cells in both undifferentiated samples and in plant and animal tissues. Complementation of the betalain biosynthetic pathway in plants and the study of the fate of betaxanthins added to the diet of model organisms benefiting from microscopy filters readily available for the GFP technology.

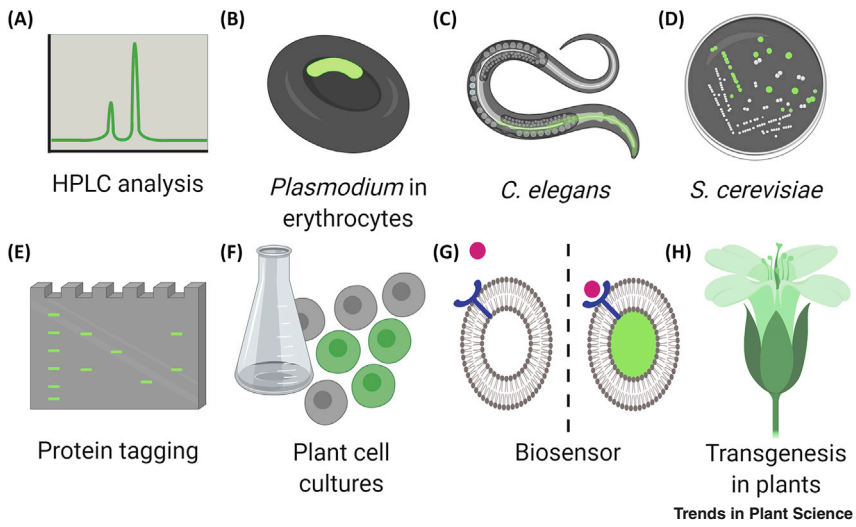

Figure 4. Schematic Overview of the Biotechnological Applications Developed from Betalains' Fluorescence.

Fluorescence can be used for the improvement of detection procedures of the pigments separated by analytical techniques such as high-performance liquid chromatography (HPLC) (A) [30]. Specific betalains are able to stain *Plasmodium falciparum* inside erythrocytes, which allows fluorescent detection of the parasite (B) [78]. Fluorescence makes betalains visible in the digestive tract of model animals as *Caenorhabditis elegans*, demonstrating ingestion and stability of bioactive pigments (C) [21]. Increased fluorescence due to betaxanthins can be linked to enhanced activities of enzymes of biotechnological interest, allowing high-throughput screening techniques in organisms such as *Saccharomyces cerevisiae* (D) [81]. Macromolecules condensed with one molecule of betalamic acid become fluorescent in a tagging procedure suitable for proteins in solution and in electrophoresis gels (E) [42]. Increased production of betalains in plant cell bioreactors can be specifically and easily followed through fluorescence (F) [77]. Fluorescence of betaxanthins is a suitable output signal for virtually any biosensor provided that betalamic acid production is engineered (G) [90]. The success of transgenesis in plants can be ascertained by betaxanthin fluorescence when using genes for betalamic acid-producing dioxygenases (H) [84]. This figure was created using BioRender (https://biorender.com/).

Visualization of Natural Betalains *In Vivo* and *In Vitro*

The first descriptions of betaxanthins' strong fluorescence accompanied spectral characterization with the visualization of different pigments in the physiological environments within the plants containing them. This happened for *P. grandiflora*, *L. productus* [7], and *M. jalapa*, where an inner filter effect with betacyanins was characterized under physiological conditions [8]. Visualization was based on microscopy filter cubes used for GFP in epifluorescence microscopes and the use of the 488 nm line of Krypton-Argon ion lasers in confocal instruments. Images of fresh samples were obtained by recording light emitted in the 500–525 nm range of wavelengths. Cells were shown with vacuoles occupying most of the cell space and with nonfluorescent black spots corresponding to nuclei. In addition, junctions between cells were visualized more clearly than with the brightfield technique [7].

Further research involved visualization under the same system for the plant *Portulaca oleracea* [73]. In this case, fluorescence in yellow petals is mainly due to miraxanthin V. The presence of multiple shades in flowers allowed the use of violet and white controls with betacyanins and without any pigment, respectively. In this species, light emission was not only recorded in yellow petals but was also detected in pistils, where the same betaxanthin was also described.

A recently described source of betaxanthins is the pseudo-cereal quinoa (*C. quinoa*), where pigments can be found in edible yellow and orange grains [13]. The presence of multiple pigments in the outer layer makes the grains of this crop fluorescent. This implies that there are glowing yellow quinoa grains, while white, red, or black grains do not fluoresce. Macroscopic images can be obtained

with suitable macroscopes under blue light stimulation [13]. The pigments responsible in this case are mainly dopaxanthin and miraxanthin V.

Fluorescence of betaxanthins has also been applied to the determination of the pigments as a function of storage conditions in foods [74]. Time-resolved fluorescence has been applied to the study of raw beet roots that have been stored under vacuum and refrigerated for up to 41 days. The proof of concept, based on fluorescence decay, proposes the possibility of using this noninvasive technique to study the evolution of betalains in foods [74,75]. The addition of betaxanthins as a dye to transform dim objects into fluorescent is also possible, as has been demonstrated for wool [76]. Indicaxanthin was used to transform white fabrics into yellow ones by adding the characteristic fluorescence of betaxanthins. In this case, maximum wavelength for emission was recorded as 576 nm. Although fluorescence was affected by light exposure, it was resistant to washing and maintained spectral properties and intensity [76].

The use of fluorescence as an imaging technique to identify the presence of betaxanthins in plant tissue has now been extended to betalain-producing cell cultures. This is the case of cell lines developed from *C. argentea* [77]. Production of the highly antioxidant diphenolic betaxanthins derived from L-DOPA and dopamine was readily followed by the presence of yellow coloration and green fluorescence with blue light stimulation under the same systems developed for tissue visualization. The existence of fluorescence and color correlated with the chromatographic identification and quantification of the pigments.

Fluorescent Detection of Malaria-Infected Erythrocytes

The coumarinic betalain BtC is a semisynthetic betaxanthin which behaves exactly like its natural analogs [78]. Like other betaxanthins, it was synthesized from betalamic acid released from betanin [28] after condensation with a particular amine: 7-amino-4-methylcoumarin. Condensation with this molecule provided interesting properties to the resulting betaxanthin: it was able to pass through the membrane of erythrocytes and to attach to the parasite *Plasmodium falciparum* inside the blood cells. As a betaxanthin, it is water soluble and is a fluorescent molecule, with fluorescence spectra with a maximum excitation wavelength of $\lambda = 520$ nm and emission with a maximum at $\lambda = 570$ nm. This means that BtC is a fluorescent probe able to stain malaria-infected erythrocytes [78]. BtC selectively accumulates within the infected cells at the point of the living parasite localization. The semisynthetic betaxanthin was later characterized in depth and derivatized as its trifluoromethyl analog, cBeet151 [33]. The characterization of the photophysical properties of the molecule and the two-photon absorption process demonstrated that the electron density migrates from the coumarinic to the betalainic moiety upon $S_0 \rightarrow S_1$ excitation (Figure 2).

The novel methodology created based on betaxanthin fluorescence requires less than 5 minutes of incubation and allows live-cell imaging with minimal cell manipulation. Malaria is a potentially deadly illness caused by the *Plasmodium* and is easily transmitted by *Anopheles* mosquitoes, mainly in wide areas of Africa, where it affects millions of people. The development of a quick and inexpensive method to detect malaria is of high medical relevance. The synthetic betaxanthin created and its quick fluorescence detection by means of microscopy may help in the important task of diagnosis by detecting the *Plasmodium* before the etiology of the illness gets severe.

Fate of Betalains Added to the Diet in the Animal Model *C. elegans*

There is growing interest in the food and pharmaceutical industries for betalains, due to their biological activities and health-promoting potential [22]. They have been described in the prevention of tumors in mice and their addition to the human diet promotes the protection of oxidative stress in blood cells [79]. Maximum plasma concentrations are reached 3 hours after consumption, with a decline corresponding to first-order kinetics.

Due to the interest in the bioactivities, bioavailability, and stability of betalains after ingestion, the animal model *C. elegans* has been used to study the effect of betalains in the diet [21]. A significant

reduction of the oxidative stress *in vivo* and the extension of the life-span of the animal have been demonstrated. The transparent body of the small nematode made it possible to follow the light emitted by fluorescent betaxanthins. Using the same filter cubes applied for plant material, it was possible to demonstrate the ingestion of the pigments by detecting green fluorescence throughout the digestive tube. This discriminated ingestion against cutaneous absorption and established a methodology to follow the stability of betaxanthins within individual animals and their effect on the health-promoting effects of the pigments. The fluorescence signal was quantified and correlated to the amount of betaxanthins detected by an alternative chromatographic method [21].

Monitoring Enzyme Activities

Continuous spectroscopic assays are useful to follow enzyme activities with high precision. Moving from absorbance to fluorescence-based assays allows increases in sensitivity and specificity, both by following the appearance of fluorescent products or by measuring the reduction of a fluorescent substrate. In addition, the accumulation of betaxanthins as fluorescent final products has been used to indicate the activity of enzymes *in vitro* and *in vivo* with a variety of different applications.

Following Oxidative Enzymes: Tyrosinase

By using betaxanthins as enzymes substrates, the activity of oxidases has been followed by a continuous fluorescent method [73]. Di-hydroxylated betaxanthins are highly antioxidant molecules due to the presence of the catecholic substructure [80]. This structure also means that the betaxanthins are substrates for oxidase enzymes like tyrosinases or peroxidases [27], while maintaining the fluorescent properties common to all betaxanthins. Oxidation generates a betaxanthin-quinone able to rearrange to leuko forms after intramolecular nucleophilic additions. The resulting compounds present highly limited fluorescence [73].

The methodology used avoids the interference of nonfluorescent molecules that might be present in extracts or formulations. Spectral changes upon enzymatic extract addition were recorded, showing the decrease of fluorescence while the substrate is converted into products. Despite the reduction in fluorescence intensity, spectral shapes and maximum wavelengths barely vary during the enzymatic activity. Selected wavelengths for the continuous recording were λ = 465 nm for excitation and λ = 512 nm for emission [73]. These wavelengths are expected to fit with the maximum signal in the spectra of most betaxanthins and are thus of use in the evaluation of the transformation or degradation of other pigments by chemical or enzymatic means.

Enhancing Drug Production in Yeast: Tyrosine Hydroxylase

Labeling of enzymatic activities through betaxanthin fluorescence is possible, not only for the transformation of the pigments. Any reaction yielding betalamic acid will promote its condensation with amines or amino acids to form the corresponding betaxanthins. Thus, the high fluorescence of the yellow pigments can be used as a suitable signal of application in screening techniques [81,82].

The production of benzylisoquinoline alkaloids in yeast relies on the use of multiple enzymes cloned from the producing plants [83]. Like betalains, alkaloids derive from L-DOPA, which is transformed in multiple steps into (*S*)-reticuline, considered the starting point for a wide variety of drugs, including codeine, morphine, and other opioid drugs [81]. However, the yeast *Saccharomyces cerevisiae* is able to transform simple sugars added to the growth medium and to synthesize L-tyrosine, the dehydroxylated precursor of L-DOPA, but it does not accumulate L-DOPA itself. Thus, engineered yeasts are able to produce L-tyrosine from sugars and to produce drugs as final products from added L-DOPA [83]. There was a missing enzymatic step between these two pathways that prevented the obtention of opioids from sugars in yeast. Filling this gap meant finding a suitable tyrosine hydroxylase activity able to convert L-tyrosine into L-DOPA without further oxidizing it to the quinone.

Because the final product of this reaction is L-DOPA, it is possible to sensor L-DOPA production by adding to the system a 4,5-DOPA-extradiol-dioxygenase enzyme (DODA). This is able to convert

L-DOPA into betalamic acid, which ultimately yields betaxanthins [81]. The dioxygenase enzyme used was the sequence from *M. jalapa* and fluorescence of betaxanthins was used then to identify L-DOPA-producing enzymes using L-tyrosine as a substrate. Furthermore, massive mutagenesis studies were performed to improve the enzyme activity because the screening process detects enhanced hydroxylating activities present in single colonies by measuring increased fluorescence [81].

The same screening application has been extended to the discovery of strains of *S. cerevisiae* producing L-tyrosine with high yields [82]. In this case, the fluorescence of betaxanthins is the signal used in the measurement of the production of L-tyrosine in yeasts from a mutant library obtained by random mutagenesis. Betaxanthin production from L-tyrosine was achieved by transformation of the wild type *S. cerevisiae* strain with the genes encoding L-tyrosine-hydroxylase and 4,5-DODA. When high yields of L-tyrosine are produced, the hydroxylase converts it into L-DOPA, which is the substrate of DODA. Then betalamic acid is formed and condenses with amino acids and amines, yielding betaxanthins, which are yellow in color and highly fluorescent. This fluorescent screening technique detected yeast mutants with high production of L-tyrosine.

The combination of these two activities in yeast (high-yield L-tyrosine production [82] and L-tyrosine hydroxylation to L-DOPA [81], both screened thanks to betaxanthin formation) may result in an enhanced production of bioactive alkaloids. The betaxanthin fluorescence signal in microorganism colonies helps to assess the phenotype of interest and makes the development of high-throughput screening strategies simple and inexpensive. Thus, betalain fluorescence discovered in flowers of the Caryophyllales helps in the production of drugs in yeast cultures.

Reporter Activity in Transgenesis: 4,5-DOPA-Extradiol-Dioxygenase

Fluorescence is a specific signal selected to measure genetic transformation of plant models and industrial crops with the capability to produce betalains. Betalains are phytochemicals with biological activities and health-promoting potential. However, natural edible sources are mainly restricted to beets, cactus pears, and Swiss chard, with few alternative sources possible [22]. This has promoted interest in the generation of edible betalain-producing plants.

Transformation of the plant model *Arabidopsis thaliana* with the 4,5-DOPA-extradiol-dioxygenase from *Amanita muscaria* demonstrated the feasibility of the transgenic approach to expand the array of betalain-producing plants [84]. Positively transformed plants resulted in the accumulation of betaxanthins in flowers and seedlings of the plant. The fluorescent properties of betalains allowed the visualization of cells in these structures. Equivalent results supported by fluorescence microscopy showed dioxygenase activity in transient expression experiments in petals of *Antirrhinum majus* transformed with the enzymes from *A. muscaria* and *P. grandiflora* [84]. The hydroxylation of tyrosine to form L-DOPA by the specific cytochrome CYP76AD5 from *B. vulgaris* was also demonstrated by betaxanthins' fluorescence in petals of *A. thaliana* [85]. This was possible after coexpression with the 4,5-DODA from *P. grandiflora*. Furthermore, studies on different types of dioxygenases and their classification as betalamic acid-forming enzymes have also been helped by the fluorescence of betaxanthins in transient expression experiments in *A. majus* petals [86]. Different dioxygenases from *B. vulgaris* and *Parakeelya mirabilis* were used and positive activity was accompanied by fluorescence of petal cells under blue light stimulation. Results in this case correlated with transcript abundance in betalain-accumulating tissues of the original plant [86].

By applying a similar approach, plants of agronomic and alimentary interest have finally been developed. This is the case for tobacco (*Nicotiana tabacum*), where metabolic engineering helped in the final elucidation of the biosynthetic pathway of betalains [87]. Fluorescence was the signal used to ascertain *in situ* the presence of betaxanthins and thus the presence of active forms of the *B. vulgaris* enzymes inserted in the leaves of transgenic tobacco. Also tomatoes (*Solanum lycopersicum*), eggplants (*Solanum melongena*), potatoes (*Solanum tuberosum*), and petunias (*Petunia* × *hybrida*) have been engineered to accumulate betalains and transformation was ascertained by the

presence of pigments [88]. Fluorescence of betaxanthins makes the tissues containing them fluorescent under blue light, as detected for tobacco flowers. Betaxanthins detected in this case were vulgaxanthin I, vulgaxanthin III, indicaxanthin, and miraxanthin III. All these plants expressed active 4,5-DODA from *B. vulgaris* and thus were able to form betalamic acid [89], which, in turn, condensed with available amines to produce the corresponding betaxanthins [88]. The presence of betalains changes the color of the vegetables developed and, in addition, enriches plants with their health-promoting activity.

In plant transformation, fluorescence demonstrates the presence of the transgene and the production of betaxanthins. The microscopy and visualization techniques used in this application are analogous to those established for plants naturally producing betalains in the Caryophyllales [8]. Fluorescent food has thus finally been created by the accumulation of betaxanthins in transgenic plants.

Protein Labeling

Betalamic acid is able to react with virtually any free amine available. This leads to the wealth of pigments present in nature but also to the formation of the semisynthetic betalains described. One notable advance in this process has been to use free amine groups present in proteins to add a molecule of betalamic acid. This results in the synthesis of the so-called yellow protein-betaxanthins [42]. Spectral properties of the molecules are analogous to betaxanthins of low molecular weight and are fluorescent. Spectral shapes are equivalent and present maximum excitation wavelength of 476 nm and a maximum emission centered at 551 nm. The proteins studied were albumin, ovalbumin, and trypsin [42], but the phenomenon might well be generalizable to any protein, as demonstrated by the use of standard molecular weight markers, where all proteins turned into protein-betaxanthins. This reaction results in a labeling procedure where proteins incorporating one molecule of betalamic acid turned fluorescent. Again, conventional devices already developed for the visualization of fluorescein or GFP as Typhon fluorescence scanners or ImageQuant CCD cameras can be used to reveal the presence of proteins tagged with betalamic acid.

A step forward in this procedure was to substitute purified betalamic acid as the labeling reagent with red beet root juice with minor purification [42]. After a simple degradation procedure involving *in situ* alkalization and neutralization, proteins in electrophoresis gels can be stained and are ready for visualization. Red beet root juice is thus an unexpected but reliable staining agent yielding fluorescent proteins at low cost.

Output Signal in Biosensors

Fluorescence of betalains provides a reliable and robust signal, which is easily detected by conventional fluorescence apparatus. For this reason, it is a suitable candidate signal for use in biosensors, as demonstrated by the development of whole-cell sensors for the analysis of environmental copper [90]. By using the bacteria *Cupriavidus metallidurans* and *Ralstonia eutropha*, it was possible to transform a copper-sensing signal mediated by a kinase into protein expression and light emission mediated by betaxanthins. This was done studying the effect of different promoters and linking the receptor to the expression of the enzyme 4,5-DODA from *M. jalapa*. This way the bacteria were able to synthesize betalamic acid, which then condensed with amines to form the corresponding fluorescent betaxanthins [90]. This fluorescence was the signal measured, which turned out to be dependent on the concentration of copper. Application of this sensor is intended for environmental samples, including freshwater and tap water.

The approach developed is not restricted to metals and opens a wide field of new applications for betaxanthins. The original work is a neat demonstration that betaxanthin fluorescence can be used as the output signal of virtually any sensing device, insofar as an inducible promoter can activate the expression of a 4,5-DOPA-extradiol-dioxygenase in the presence of L-DOPA. This will indefectibly lead to the production of betaxanthins and to the emission of green fluorescent light.

Synthesis of Tailor-Made Probes and Future Prospects

Synthetic betalains can be tailor-made in order to fit specific requirements. The success of the design of a betaxanthin probe able to pass through blood cells membranes and to stain *P. falciparum* is a good example [78]. The amine group used in the formation of the imine pigment can be chosen or derivatized by the researcher while maintaining the betalamic acid structure intact. It is only a matter of time before novel capabilities can be added to betaxanthins by modifying this variable substructure. Possibilities are wide, as evidenced by the current works, but the success of this approach will depend on the capability of producing betalamic acid in sufficient amounts as a starting molecule.

Betalamic acid can be produced synthetically in a chemical and complex process [91] or obtained from pigments extracted from plants after alkaline degradation [92]. In both cases yields are low. The acid can be isolated from betanin after cleavage of the Schiff base by extraction with ethyl acetate [93]. This process also involves low yields but the amounts and purity obtained are enough to generate betaxanthins suitable as HPLC standards. After the same degradation process, betalamic acid can be purified by anion exchange chromatography if the pH of the solution is maintained high [94]. In this case, the process can be scaled-up to yield sufficient amounts to characterize the properties and bioactivities of the isolated molecule and to produce synthetic betalains [28]. For small-scale synthesis, a betalamic acid-derivatized matrix has been developed that makes affordable the one-step synthesis of individual betalains by simply adding the selected amine [95].

Current research on the betalamic acid-forming enzymes discovered in the biosynthetic pathway of betalains has opened a new way to produce the molecule. Enzyme assays with 4,5-DODA show that, after the cleavage of the L-DOPA aromatic ring, intramolecular rearrangement of the generated 4,5-secodopa intermediate occurs and betalamic acid is formed. This had been demonstrated for the enzymes from *A. muscaria* and *P. grandiflora in vivo* [96]. Now enzyme-mediated formation of betalamic acid has moved from plants to *in vitro* in enzyme assays after the heterologous expression of the enzymes from *M. jalapa* [97], *B. vulgaris* [89], and even with bacterial enzymes from *Escherichia coli* [98] and *Gluconacetobacter diazotrophicus* [99]. The application of stable enzymes to microbial bioreactors [100,101] anticipates the production of tailor-made betalains in high yields and with novel capabilities.

To date, fluorescence of betaxanthins has been used to identify and characterize proteins, to stain microscopic parasites, to follow enzyme activities, and to locate pigments in the digestive tract of model organisms, in addition to ascertaining the production of pigments both *in vivo* and *in vitro*, both in natural and transgenic plants (Figure 4). Possibilities are wide, as evidenced by current works, and synthetic betalains can be tailor-made to fit specific requirements. The betaxanthins obtained may incorporate novel capabilities, depending on their substructure, but they will maintain the same fluorescent properties described for the natural ones insofar as the betalamic acid moiety remains unaltered (Figure 1E). It can be anticipated that the methods already developed for detection, microscopy visualization, and high-throughput screening based on betaxanthin fluorescence reviewed in this work will be straightforward to apply to novel tailor-made molecules.

Concluding Remarks

The discovery of fluorescence in betalains opened up new lines of research and applications of the plant pigments. Flowers and plant structures containing them are naturally fluorescent and emit green light upon excitation. Studies performed soon characterized structure–activity relationships in the pigments and explained the photophysics of the phenomenon. The unexpected finding that betaxanthins are able to emit green fluorescence, combined with the active research on the key enzyme producing betalamic acid in plants, generated the interesting possibility of direct fluorescent visualization in transgenic organisms. Microscopy, screening, and high-throughput procedures were developed for multiple applications and benefit from an easily measured signal. Current applications of betalain fluorescence, comprehensively summarized in this work, far exceed the expected applications considered when the first betalain-containing fluorescent flowers were described (see

Outstanding Questions

What is the relevance of green flower fluorescence due to betaxanthins and the contrasting patterns due to violet betacyanins in biosignaling?

Were betalains selected preferentially over anthocyanins in the evolution of the Caryophyllales order due to color and fluorescence properties? Is there any evolutionary advantage for these plants or is it a side-effect of chemical structures that were selected by other factors?

Given the new insights into the photophysics phenomenon, to what extent can fluorescence of betalains be enhanced in order to produce increasingly sensitive detection techniques?

Can bioinspired, tailor-made betalains exploit specific recognition interactions to be used for tagging molecules or structures *in vivo*?

Outstanding Questions). Fourteen years after the initial publications, it is clear that betalains provide more than colors.

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The authors declare no competing financial interest.

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