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Review

Autofluorescence: Biological functions and technical applications

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ABSTRACT

Chlorophylls are the most remarkable examples of fluorophores, and their fluorescence has been intensively studied as a non-invasive tool for assessment of photosynthesis. Many other fluorophores occur in plants, such as alkaloids, phenolic compounds and porphyrins. Fluorescence could be more than just a physicochemical curiosity in the plant kingdom, as several functional roles in biocommunication occur or have been proposed. Besides, fluorescence emitted by secondary metabolites can convert damaging blue and UV into wavelengths potentially useful for photosynthesis. Detection of the fluorescence of some secondary phytochemicals may be a cue for some pollinators and/or seed dispersal organisms. Independently of their functions, plant fluorophores provide researchers with a tool that allows the visualization of some metabolites in plants and cells, complementing and overcoming some of the limitations of the use of fluorescent proteins and dyes to probe plant physiology and biochemistry. Some fluorophores are influenced by environmental interactions, allowing fluorescence to be also used as a specific stress indicator.

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1. Fluorescence, the basics

1.1. Basic aspects

Luminescence refers to the spontaneous emission of light by atoms or molecules as a consequence of the deactivation from an electronic excited state to the ground state. If the radiative

Abbreviations: F, fluorophore; FRET, Förster resonance energy transfer; ProtoIX, protoporphyrin IX; Q, quencher.

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deactivation occurs between electronic states of the same multiplicity (singlet–singlet, triplet–triplet) the process is called fluorescence, whereas phosphorescence is assigned when the emission occurs between states of different multiplicity. Because the electronic spin cannot change during a spectroscopic transition, phosphorescence is a forbidden process and more difficult to be experimentally observed than fluorescence. Non-radiative deactivation processes from the excited state, in which the excitation energy is generally dissipated as heat, are also possible: internal conversion, if the involved electronic states have the same multiplicity; or intersystem crossing when the process implies a change in the electronic spin. All of these deactivation processes are competitive with each other, and the predominant deexcitation mechanisms should be those with the highest deactivation rate constants [1].

The general excitation–deactivation processes that take place in the electronic excited states are illustrated in the Jablonski diagram (Fig. S1). Normally, a molecular system in the ground state has all the electronic spins paired, and it is a singlet (S_0). Depending on the excitation wavelength, the photoexcitation (excitation by absorbing light) can populate different singlet electronic-vibrational excited states (S_1(v), S_2(v), ...), but rapidly loses the excess excitation energy by very-fast (in the femtosecond time scale) internal conversion and/or vibrational relaxation mechanisms to reach the lowest electron-vibrational excited state (S_1). During the lifetime of S_1 (in the time scale of nanosecond), its deactivation to S_0 can be due to fluorescence (with a k_{fl} rate constant) or to internal conversion (k_{ic} rate constant). Since a singlet excited S_1 state has associated a triplet excited T_1 state with lower energy (Hund rule), S_1 can also deactivate to T_1 via an intersystem crossing process (k_{isc} rate constant). Finally, T_1 can return to the ground state by phosphorescence and/or intersystem crossing processes. Since the energy of T_1 excited is lower that the corresponding S_1, the T_1–S_0 energy gap is lower than that between S_1 and S_0 states, and phosphorescence should emit at longer wavelengths than the fluorescence emission band. Besides, the excitation process (from S_0 to S_n) requires equal or higher energy that the fluorescence deactivation (from S_1 to S_0), and the fluorescence band is placed at lower energies than the absorption (excitation) band, and the spectral shift between both bands is the Stokes shift [1].

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After all these considerations, a molecular system should be highly fluorescent (fluorophore) when the radiative deactivation from S_1 is much higher than the non-radiative processes (k_{nr} rate constant, including both S_1–S_0 internal conversion and S_1–T_1 intersystem crossing, $k_{nr} = k_{ic} + k_{isc}$). Indeed, a molecular system is potentially fluorescent for a high k_{fl} value (allowed radiative transition such as in aromatic compounds), and low k_{ic} (rigid systems, where the thermal energy dissipation becomes more difficult) and k_{isc} (absence of halogen atoms, the so-called heavy atom effect, since halogens favour the change of the multiplicity by spin-orbit coupling) values. The fluorescence quantum yield, defined as the probability to emit a photon per absorbing photon, is given by the ratio between the fluorescence (k_{fl}) over all the deactivation processes from S_1 ($k_{fl} + k_{nr}$).

We consider here only those fluorophores that emit light in the visible range (roughly from 400 nm to 700 nm) with a substantial quantum yield. All living cells are to some extent fluorescent, because of the presence of some fluorescent molecules essential for life, including pyridine nucleotides and flavins. Most of the natural fluorophores are excited by UV radiation, and, when present at significant concentrations, can be easily visualized with the naked eye by a suitable choice of the excitation wavelengths with a proper light filter, observing the fluorescence through another light filter that will allow the passage of the fluorescent light and block completely the excitation wavelengths. The term "autofluorescence" is used to distinguish the fluorescence emitted by endogenous fluorophores in biological samples from that generated by the use of exogenous fluorescent dyes (not naturally present in a tissue) or genetically engineered fluorophores (e.g., green fluorescent protein). Light generated by chemical reactions (chemiluminescence) in plant cells is not related to autofluroescence. The present review focuses on possible biological functions of autofluorescence phenomena in plants, and how the study of in vivo fluorescence can be a useful tool in studying plant physiology and biochemistry.

1.2. Factors affecting fluorescence quantum efficiency and spectrum

The previous section introduces the unimolecular photophysical processes for the deactivation of the electronic excited states, describing the fluorescence ability of a fluorophore (F) in its surroundings. However, the presence of a second molecule can decrease the fluorescence intensity. This bimolecular process is called fluorescence quenching, and the loss in the fluorescent capacity is dependent on the quencher (Q) concentration. This quenching can be static or dynamic. Static quenching generally refers to the formation of a non-fluorescent F–Q complex in the ground state (Fig. 1). The quenching does not induce a new deactivation process in the excited state, but reduces the proportion of absorbing photons by the fluorophore in the excitation process, decreasing the fluorescence intensity without affecting the fluorescence lifetime. The apparent reduction in the fluorescence quantum yield can be corrected if the proportion of the absorbing photons by the fluorophore is taken into account. On the other hand, dynamic quenching occurs when a new deactivation process from S_1 state is induced by the presence of the quencher. This dynamic process competes with the fluorescence emission of the fluorophore, and both the fluorescence quantum yield and lifetime decrease by increasing the quencher concentration. The process is said to be dynamic because during the lifetime of the fluorescent state both F and Q can be moved to reach each other.

Several mechanisms can contribute to the dynamic quenching. One is an inelastic collision between F (in its fluorescent excited state) and Q, in which the excitation energy is transfered to Q and/or to the surroundings. In this case the quenching requires contact between both species, and the corresponding rate constant depends on the solvent viscosity. A wide variety of chemicals can quench fluorescence at specific wavelengths, including molecular oxygen, halides, aromatic and aliphatic amines, purines, pyrimidines, acrylamide, disulfides, nitric oxide, imidazole or hydrogen peroxide, and others [1]. The reaction between some quenchers and fluorophores is compound-specific, e.g.: FAD and NAD(P) fluorescence can be quenched by purines such as adenosine and adenine, while chloride ion is a well known quencher for quinine [2]. A second mechanism is the Förster resonance energy transfer (FRET), where the excitation energy is transfered from the excited state of F to Q. This mechanism takes place via dipole–dipole interaction, and does not require molecular contact between the involved species. Indeed, it is known as a long-range distance (in the order of 100 Å) process, but it requires a spectral overlapping between the F fluorescence band and the Q absorption band. Other mechanisms, such as a photochemistry reaction, complex formation in the excited state or a charge transfer between the excited state of F and Q, are also possible. In the latter case, the quenching constant would depend on the ionization potential of the electron donor and the electron affinity of the acceptor.

Independent of the mechanisms, when a quencher produces a new fluorescent species the process induces the observation of a new emission spectral band to the detriment of the original