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A quantitative description of fluorescence excitation spectra in intact bean leaves greened under intermittent light

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Abstract

We present a simple approach for the calculation of in vivo fluorescence excitation spectra from measured absorbance spectra of the isolated pigments involved. Taking into account shading of the pigments by each other, energy transfer from carotene to chlorophyll *a*, and light scattering by the leaf tissue, we arrive at a model function with 6 free parameters. Fitting them to the measured fluorescence excitation spectrum yields good correspondence between theory and experiment, and parameter estimates which agree with independent measurements. The results are discussed with respect to the origin and the interpretation of in vivo excitation spectra in general.

Abbreviations: a – absorptance (=1-transmittance); A – absorbance (=−log transmittance); Chl – chlorophyll; PS – photosystem; λ – wavelength

Introduction

Fluorescence excitation spectroscopy is a well-established method for the identification of pigments involved in energy transfer, and for the estimation of energy transfer efficiencies among them – usually by comparing excitation and absorption spectra (e.g., Goedheer 1969, Gugliemelli et al. 1981, Kramer et al. 1981, Plumley and Schmidt 1987). On this basis, excitation spectra may be used for monitoring energy transfer paths, which may be interpreted in terms of the functional organisation of the pigment systems (Siefermann-Harms 1985, 1987, Brody 1988).

The method suffers, however, from a major drawback when applied to intact biological systems because the leaf anatomy and the shading of the active pigment by itself and by accompanying pigments tend to distort the spectra or even shift their maxima (for a review see Butler 1972 or Schäfer et al. 1983).

Dealing with these complications, we present here a simple model for the description of fluorescence excitation spectra as applied to partially greened bean leaves. The results will yield some general insight into the origin of such spectra and facilitate their interpretation.

Materials and methods

Bean seeds (*Phaseolus coccineus var.* Preisgewinner) were planted in moist vermiculite-perlite and grown for 12 days in the dark followed by 9 days of intermittent illumination (100 s light al-

ternating with 100 min dark). Illumination was with a common 60 W-bulb mounted at a distance of 50 cm from the leaves yielding a radiant flux density of about 15 W/m^2.

For pigment determination, 1 g of leaves was homogenised in a cold mortar together with 1 g of $CaCO_3$. Pigments were extracted with pure acetone and, after removal of acetone by vacuum-evaporation, dissolved in a small volume of diethylether. Pigments were separated using the thin-layer chromatography developed by Egger (1962), which does not separate β, ε-isomers. Carotene was determined in chloroform, xanthophylls in ethanol, and Chl *a* and *b* in acetone, using published extinction coefficients (Hager and Meyer-Bertenrath (1966) for carotenoids, Ziegler and Egle (1965) for the chlorophylls).

For absorbance and fluorescence measurements one half of a primary leaf was mounted onto a frame (1.5 × 4 cm) which was positioned diagonally in a macro-cuvette. To prevent desiccation of the sample a piece of blotting-paper was placed at the bottom of the cuvette. Measurements were performed at 25°C with pigments solutions, and at room temperature with leaf material, respectively.

Absorbance spectra were recorded using an Aminco DW-2a spectrophotometer on line with a microcomputer (North Star Computer HORIZON) and digitised with 12 bit resolution (Software: Arete-Systems, California). Fluorescence excitation spectra were recorded at room temperature using a JY 3 D spectrofluorometer equipped with a 150 W xenon lamp on line with our microcomputer. Measuring beam and excitation light reached the leaf surface at an angle of 45°.

Fluorescence detection was from the leaf surface at right angles with the incident light. A blue glassfilter (520 ± 120 nm, unspecified filter of Schott, Mainz) was mounted between excitation monochromator and the leaf sample, and a red glass filter (RG 695, Schott, Mainz) placed between sample and emission monochromator. The photon flux density of incident light (band width: 4 nm) at the leaf surface was between 17 and 33 $\mu E/(sec\ m^2)$, changing smoothly with the wavelength. Prior to the measurements, samples were exposed to the excitation beam for 30 s. Fluorescence detection was at 690 nm with a band-width of 10 nm. Excitation spectra were quantum-corrected with the rhodamine B quantum counter method using 0.3% rhodamin B dissolved in ethyleneglycol.

Spectra of extinction coefficients

The absorbance spectra entering the simulation of fluorescence excitation spectra were recorded from carotene in chloroform and violaxanthin in ethanol, both isolated from *P. coccineus* and separated according to Egger (1962). Chl *a* (solvent: acetone) and lutein (solvent: ethanol) were extracted from leaves of *Nicotiana tabacum*, kept in the dark for 12 h before isolation followed by separation as described in Egger (1962). Lutein was considered to be pure since predarkened tobacco plants show very low concentrations of the co-migrating isomer zeaxanthin (Meck and Strasser 1984).

Zeaxanthin, the absorbance spectrum of which was needed for determining the in vivo position of violaxanthin absorbance (Fig. 1), was enriched in etiolated *Phaseolus vulgaris* as described elsewhere (Pfündel and Strasser 1988). Pigment chromatography was carried out using the alkaline thin-layer plate developed by Hager and Meyer-Bertenrath (1966), which separates β, ε-isomers. The absorbance spectrum of zeaxanthin was recorded in an ethanolic solution.

Each absorbance spectrum was normalised by multiplying the whole curve by the quotient of its corresponding extinction coefficient and its absorbance at the wavelength of the extinction coefficient (those extinction coefficients used for pigment determination in the case of the carotenoid spectra, and the extinction coefficient of the soret band determined by Mackinney (1940) for Chl *a*). In order to obtain spectra which can be taken as spectra of extinction coefficients in situ, we had to shift the normalised spectra to their putative in vivo positions. As a reference, we selected the long-wavelength maxima for the carotenoid species, and the soret band for Chl *a*. The respective in vivo positions were taken to be 440 nm for Chl *a* (Goedheer 1969, Plumley and Schmidt 1987) and 495 nm for carotene (Goedheer 1969, Ji et al. 1968).

The position of the long-wavelength violax-

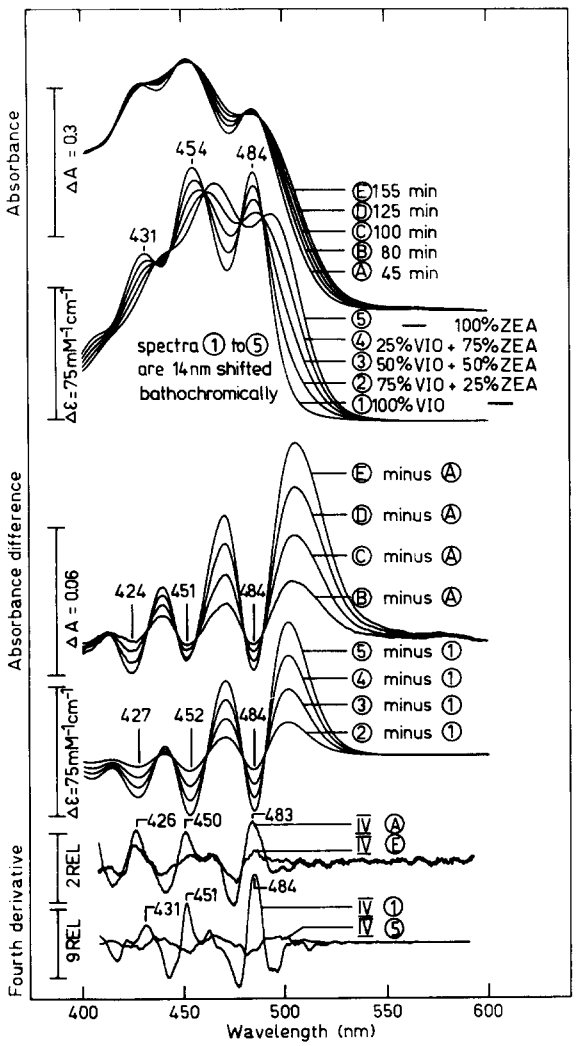

Fig. 1. Determination of absorbance maxima of violaxanthin in vivo. Curve A to curve E shows the shift in absorbance spectra in the course of violaxanthin de-epoxidation in an etiolated leaf (cf. Pfündel and Strasser 1988). The spectra were recorded at the indicated times after vacuum-infiltration with a pH 5 buffer containing 80 mM of ascorbate. From the absorbance spectra difference spectra (curve B minus A to curve E minus A) and fourth derivative spectra (curve IV A and curve IV E) are calculated. In comparison, bathochromically shifted absorbance spectra of equimolar ethanolic solutions of violaxanthin (curve 1) and zeaxanthin (curve 5) as well as absorbance spectra of mixtures of these solutions (curve 2 to curve 4) are shown. The minima of the resulting difference spectra (curve 2 minus 1 to curve 5 minus 1) can be traced back to the corresponding absorbance maxima of violaxanthin. Likewise, the maxima of the fourth derivative spectrum of violaxanthin (curve IV 1) are related to the absorbance spectrum of violaxanthin. Since the long-wavelength peak position of isolated violaxanthin coincides with the corresponding minimum in difference spectra, the long wavelength in vivo position of the violaxanthin absorbance maximum in vivo was deduced from the corresponding minimum of the absorbance differences during violaxanthin de-epoxidation in etiolated bean leaves. To this end, we used the relation between the long-wavelength minimum of absorbance differences of equimolar solutions of violaxanthin and zeaxanthin, and the corresponding absorbance maximum of violaxanthin (Fig. 1). Since the position of the long-wavelength minimum in difference spectra of isolated pigments coincides with the corresponding violaxanthin maximum, the in vivo position of the violaxanthin long-wavelength band was taken to be identical with the corresponding minimum of the in vivo difference spectrum (484 nm, Fig. 1). The distinct maxima of the fourth derivative spectrum of etiolated leaves are related to the violaxanthin absorbance peaks since the maxima disappear during violaxanthin de-epoxidation (Fig. 1). The value of 483 nm for the long-wavelength derivative band supports the above-mentioned procedure. Since the long-wavelength band of violaxanthin in ethanol was found to be at 470 nm, the in vitro spectrum was shifted 14 nm bathochromatically. Introduction of the same shift into the spectrum of isolated lutein moves its long-wavelength band to 487 nm. This actually coincides with the long-wavelength band of xanthophylls in isolated light-harvesting complexes (Siefermann-Harms 1985, 1987), the main carotenoid of which is lutein (Siefermann-Harms and Ninnemann 1982).

Results

Pigment analysis by thin-layer chromatography reveals a high Chl *a*/Chl *b* ratio in our bean leaves (Table 1) and confirms the low Chl *b* content observed earlier in leaves greened under intermittent light (Akoyunoglou et al. 1966, Smillie et al. 1975, Siefermann-Harms et al. 1980).

spectrum was taken to be identical with the corresponding minimum of the in vivo difference spectra. The position of the long-wavelength maximum of IV A supports the above-mentioned procedure. The different scales used for the derivative spectra are due to a 4.5-fold enlargement of curves IV A and IV E relative to IV 1 and IV 5.

Table 1. Comparison of experimental and fitted values. Experimental concentrations are derived from pigment analysis by thin-layer chromatography of bean leaves greened under intermittent light for 9 days. All parameter estimates result from fitting spectra calculated according to Eq. (12) to the quantum-corrected fluorescence excitation spectra of a bean leaf (Fig. 3) taken from the same culture as was used for pigment analysis. The experimental absorbance at 440 nm of this leaf was measured using the absorbance at 560 nm as a reference. In the rightmost column, the ratios of fitted and measured values are shown

	Experimental value	Fitted value	Fitted value / Experimental value
Relative pigment concentration (mole/100 mole total)			
Chlorophyll *a*	52.6	57.7	1.10
Chlorophyll *b*	3.2	not considered	–
Carotene	7.3	3.8	0.52
Lutein	15.5	19.2	1.24
Antheraxanthin	2.5	not considered	–
Violaxanthin	15.3	19.2	1.25
Neoxanthin	3.6	not considered	–
Absorbance at 440 nm	0.85	1.25	1.47
scattering coefficient r (nm^4)	not available	3.4×10^{10}	–

The blue-green region of the absorbance spectrum of these leaves is characterised by maxima near 425, 445 and 485 nm (Fig. 2), as is already known for plants deficient in Chl *b* (Butler 1965, Goedheer 1969, Strasser and Butler 1976).

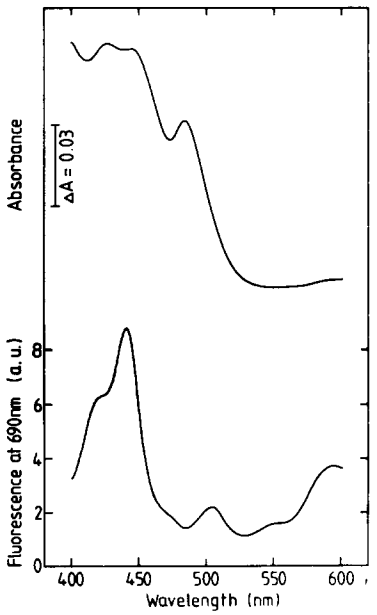

Fig. 2. Absorbance spectrum and quantum-corrected fluorescence excitation spectrum of a bean leaf greened under intermittent light. Spectra were taken from a primary leaf of a bean plant, which was germinated for 12 days in the dark followed by 9 days of intermittent illumination. Room temperature fluorescence was measured at 690 nm.

Quantum-corrected fluorescence excitation spectra of our samples only show bands which correspond to the two shorter-wavelength absorbance maxima (Fig. 2). In contrast to the absorbance curves, excitation spectra show a minimum at 485 nm and distinct maxima near 505 and 590 nm. Similar observations have been made by other authors for plants poor in Chl *b* (Goedheer 1969, Strasser and Butler 1977).

Usually, excitation spectra can be interpreted by comparing their characteristics with known absorbance bands of the pigments present. Since the fluorescence excitation spectra of intact leaves, however, poorly resemble the absorbance spectra, identification of the pigments involved in fluorescence is hardly feasible this way, even if each single absorbance peak in vivo could be traced back to a certain pigment. In particular, this is valid for the excitation maximum at 505 nm, which has no corresponding maximum in the absorbance spectrum. Consequently, the understanding of fluorescence excitation spectra requires a deeper insight into how these spectra originate from the optical properties of a leaf.

Theoretical considerations

Fluorescence excitation spectra of a dilute solution

Fluorescence emission of a dilute solution of a fluorescent pigment 1 is given by the quanta

absorbed by the pigment (E_1) times its fluorescene quantum yield (Φ_F) times a constant K depending on the fluorimeter used:

$$F_1 = E_1 \Phi_F K . \quad (1)$$

If excitation is by monochromatic light passing in x-direction through a sample of thickness d, $E_1(\lambda, d)$ is described according to the Lambert–Beer law:

$$E_1(\lambda, d) = \int_0^d \mathrm{d}E_1(\lambda, x)$$

$$= \alpha_1(\lambda)\, c_1 \int_0^d I(\lambda, x)\, \mathrm{d}x , \quad (2)$$

where $I(\lambda, x)$ means the quantum flux density as a function of wavelength λ and position x, and $\alpha_1(\lambda)$ and c_1 represent the absorption coefficient at λ and the concentration of pigment 1, respectively.

The exponential decay of $I(\lambda, x)$ depends on the absorption coefficients $\alpha_i(\lambda)$ and the concentrations c_i of all pigments i present in the sample:

$$I(\lambda, x) = I_0 \, \mathrm{e}^{-x \sum_{i=1}^{n} \alpha_i(\lambda) c_i} , \quad (3)$$

where I_0 is the intensity of incident light. Replacing $I(\lambda, x)$ in Eq. (2) by Eq. (3) followed by integration leads to:

$$F_1(\lambda) = I_0 \Phi_F K \frac{\alpha_1(\lambda) c_1}{\sum_i \alpha_i(\lambda) c_i} (1 - \mathrm{e}^{-d \sum_i \alpha_i(\lambda) c_i}) , \quad (4)$$

where Φ_F is taken to be constant as should be the case for our experimental conditions, under which induction effects can be neglected (compare Materials and Methods). Φ_F = constant is required later for the formulation of the normalised fluorescence excitation $S(\lambda)$ (see Eq. (11)).

If we now take into consideration energy transfer from non-fluorescing pigments to pigment 1, Eq. (1) has to be extended:

$$F_1(\lambda) = \sum_i E_i \Phi_{T_i} \Phi_F K , \quad (5)$$

where E_i is the number of quanta absorbed by pigment i and Φ_{T_i} represents the quantum yield of energy transfer from pigment i to pigment 1. Here, Φ_{T_i} is chosen to be one for $i = 1$, and $0 \leq \Phi_{T_i} \leq 1$ for $i = 2, \ldots, n$. Expressing E_i according to Eq. (2), one obtains:

$$F_1(\lambda) = I_0 \Phi_F K \frac{\sum_i \Phi_{T_i} \alpha_i(\lambda) c_i}{\sum_i \alpha_i(\lambda) c_i} \times (1 - \mathrm{e}^{-d \sum_i \alpha_i(\lambda) c_i}) . \quad (6)$$

Fluorescence excitation spectra of leaves greened under intermittent light

An application of Eq. (6) to intact tissue implies that light absorption can be described by the Lambert–Beer law, which requires, for instance, a homogeneous distribution of pigments. We assume, that in the developing leaves investigated here, chloroplasts are not packed near the cell wall by large vacuoles and, therefore, are distributed rather homogeneously within the cell. From the fact that chloroplasts of intermittent light plants are devoid of grana stacks (Strasser and Butler 1976, Akoyunoglou 1981), an equal pigment distribution within the plastid is inferred. For these reasons, deviations from the Lambert–Beer law due to varying transparency of the leaf (sieve effect) will not be considered in our model (but may play a significant role in other tissues).

Another problem typical of leaves is posed by reabsorption of fluorescence quanta, which tends to distort fluorescence emission spectra as well as fluorescence excitation spectra and should thus be considered when describing in vivo fluorescence. However, in our leaves the absorbance at 689 nm (maximum of fluorescence emission) measured with the 750 nm absorbance as a reference is small ($A_{689} \leq 0.15$, not shown). We infer from these observations that, in our samples, reabsorption can be neglected.

Considering only scattering events which lead to a decrease of absorption, we confine ourselves to the outermost layers of the leaf through which light passes first. In this simplified conception, scattering properties of a leaf are viewed as an optical filter transmitting an intensity $\tilde{I}_0 < I_0$ to the photosynthetic pigments. Assuming that weakening of light by scattering is describable by the general form of the Lambert–Beer law and

that light scattering in leaves can be approximately described by Rayleigh's law the transmittance $T_s(\lambda)$ is given by:

$$T_s(\lambda) = \frac{\tilde{I}_0(\lambda)}{I_0} = e^{-\lambda^{-4}r}, \quad (7)$$

where r contains the product of the scattering coefficient and the apparent thickness of the scattering layer (which is much smaller than d), and λ is the wavelength of incident light.

Consequently, $T_s(\lambda)$ is a wavelength dependent property of the leaf, which is inserted in Eq. (6):

$$F_1(\lambda) = I_0 e^{-\lambda^{-4}r} \Phi_F K \frac{\sum_i \Phi_{T_i} \alpha_i(\lambda) c_i}{\sum_i \alpha_i(\lambda) c_i} \times (1 - e^{-d \sum_i \alpha_i(\lambda) c_i}). \quad (8)$$

Classification of the dominating pigments in intermittent light leaves

To simplify calculations, only pigments contributing more than 5 mol% to the total pigment content are considered. In our samples, these are chlorophyll *a*, carotene, lutein and violaxanthin (Table 1). Since the fluorescence yield of C_{40}-carotenoids with 9 or more double-bonds is negligible (Dallinger et al. 1981, Seely and Connolly 1986), fluorecence is expected to originate from Chl *a* only. From experiments with greening bean leaves, the low Chl *b* content of which is comparable to our samples, Goedheer (1969) suggests a negligible quantum yield of energy transfer from xanthophylls to Chl *a*. An equivalent conclusion can be drawn when comparing the xanthophyll dependent absorbance maximum of intermittent light leaves at 485 nm with the corresponding minimum of the fluorescence excitation spectrum (Fig. 2). These observations may be explained by presupposing that energy transfer from carotenoids to chlorophyll requires their close proximity within a pigment-protein complex, and by taking into account that xanthophylls are mainly organised in light-harvesting complexes (cf. Siefermann-Harms 1985), which are present at low concentrations in Chl *b*-less leaves.

Photosynthetic carotenes are predominantly found in PS I and PS II (Siefermann-Harms 1985) which both have been proven to be present in plants greened under intermittent light (Akoyunoglou and Michelinaki-Maneta 1975, Strasser and Butler 1976). The yield of energy transfer from β-carotene to Chl *a* in greening bean leaves was determined to be close to 100% (Goedheer 1969), whereas a value between 50 and 60% was estimated in a barley mutant lacking Chl *b* (Kramer et al. 1981). From the above-mentioned observations we assume for our samples that only carotene is capable to transfer absorbed light energy to chlorophyll *a* and does so with an efficiency of 100%, whereas energy transfer from xanthophylls (lutein and violaxanthin) is negligible. Denoting Chl *a*, carotene, lutein and violaxanthin with indices 1 to 4, we consequently have $\Phi_{T_1} = \Phi_{T_2} = 1$, and $\Phi_{T_3} = \Phi_{T_4} = 0$.

When applying Eq. (8) to intact leaves the distribution of photosynthetic pigments between different pigment protein complexes must be considered. Since PS I contributes nothing to our room temperature fluorescence signal at 690 nm (Stahl et al. 1989), the numerator (but not the denominator) of the fraction in Eq. (8) must be corrected. To this end, we introduce c_1^{I}, c_2^{I} and c_1^{II}, c_2^{II} denoting the pigment portion associated with PS I and PS II, respectively, where $c_1 = c_1^{\mathrm{I}} + c_1^{\mathrm{II}}$ and $c_2 = c_2^{\mathrm{I}} + c_2^{\mathrm{II}}$.

In a synopsis on the pigment composition of pigment protein complexes in oxygen evolving organisms, Siefermann-Harms (1985) found comparable ratios of Chl *a* to carotene for PS I and PS II core complexes. Hence, in the case of our leaves where light harvesting complexes need not to be considered one has:

$$\frac{c_1^{\mathrm{I}}}{c_1} \approx \frac{c_2^{\mathrm{I}}}{c_2} := \frac{c^{\mathrm{I}}}{c}. \quad (9)$$

Taking into account the pigment fraction not associated with fluorescence, introducing the dominating pigments as discussed above, and replacing the absorption coefficients $\alpha_i(\lambda)$ by the more common extinction coefficients $\varepsilon_i(\alpha_i = \log_{10} e\, \varepsilon_i)$ we can rewrite Eq. (8) to yield our final model function:

$$F_1(\lambda) = I_0\, e^{-\lambda^{-4}r}\, \Phi_F\, K\left(1 - \frac{c^I}{c}\right) \times \frac{\varepsilon_1(\lambda)c_1 + \varepsilon_2(\lambda)c_2}{\sum_i \varepsilon_i(\lambda)c_i}\left(1-10^{-d\sum_i \varepsilon_i(\lambda)c_i}\right), \quad (10)$$

$$S(\lambda) := \frac{F_1(\lambda)}{I_0\, \Phi_F\, K\left(1 - \frac{c^I}{c}\right)} = e^{-\lambda^{-4}r}\, \frac{\varepsilon_1(\lambda)c_1 + \varepsilon_2(\lambda)c_2}{\sum_i \varepsilon_i(\lambda)c_i} \times \left(1-10^{-d\sum_i \varepsilon_i(\lambda)c_i}\right), \quad (11)$$

where $S(\lambda)$ is the normalised fluorescence excitation. Thus, $S(\lambda)$ is the product of T_s, the scattering function, f, the absorbance associated with fluorescence relative to the total absorbance, and a, the total absorptance ($a = 1-10^{-d\sum_i \varepsilon_i(\lambda)c_i}$):

$$S(\lambda) = T_s(\lambda)\, f(\lambda)\, a(\lambda)\,. \quad (11a)$$

Simulation of fluorescence excitation spectra

The measured spectra of ε_i (normalised and shifted to their putative in vivo-position as described in Materials and Methods) serve as an input into the model. The behavior of the model function is then determined by 6 free parameters, r, d, and $c_1, \ldots, c_4$, which could be directly used for simulation and parameter fitting. We intend, however, to compare the fitted values with quantities measured independently. As molar proportions of pigments can be determined much more precisely than their actual concentrations in the leaf, we prefer using the transformed parameters $\tilde{c}_i := c_i/c$, $i = 1, \ldots, 4$, with an arbitrary c, and the total absorbance at the reference wavelength $\lambda = 440$ nm:

$$A_{440} = d\sum_i \varepsilon_i(440)c_i = cd\sum_i \varepsilon_i(440)\tilde{c}_i\,.$$

In terms of these parameters the model function reads:

$$S(\lambda) = e^{-\lambda^{-4}r}\, \frac{\varepsilon_1(\lambda)\tilde{c}_1 + \varepsilon_2(\lambda)\tilde{c}_2}{\sum_i \varepsilon_i(\lambda)\tilde{c}_i} \times \left(1-10^{-A_{440}\cdot\frac{\sum_i \varepsilon_i(\lambda)\tilde{c}_i}{\sum_i \varepsilon_i(440)\tilde{c}_i}}\right), \quad (12)$$

and is now fitted to the experimentally measured fluorescence excitation spectrum by varying the (transformed) parameters by trial and error (empirical parameter variation). The procedure results in a model spectrum which reproduces very well the peak positions and the peak proportions of the experimental curve (Fig. 3A and B).

The simulation shown in Fig. 3 is calculated with the molar proportions of Chl *a*: carotene: lutein: violaxanthin as 15:1:5:5, the total absorbance $A_{440} = 1.25$ and the scatter coefficient $r = 3.4 \times 10^{10}$ nm^4. The relative deviation of the simulated spectrum from the experimental one, the former normalised with respect to the main peak of the latter, does not exceed 30% (Fig. 4).

Discussion

We have pointed out the discrepancy between excitation spectra of Chl *a* fluorescence and absorbance spectra in leaves which are deficient in Chl *b* due to a greening process under intermittent light (Fig. 2).

This inconsistency is resolved by calculating excitation spectra according to a model in which carotene transfers its absorbed light energy to Chl *a* with 100% efficiency and xanthophylls are incapable of such a transfer. The model, which takes as a basis light propagation according to the Lambert–Beer law and a decreased availability of exciting light due to Rayleigh scattering, reproduces the distinct in vivo maximum at 504 nm, although none of the spectra of extinction coefficients $\varepsilon_i(\lambda)$ used for the calculation has absorbance bands in this region (Fig. 3). (This observation corresponds to the above-mentioned discrepancy found for the in vivo situation.)

Our approach traces this particular excitation maximum back to the correction of the spectrum of total absorptance a by the spectrum of the absorbance fraction f which is associated with fluorescence (Fig. 3C). In other words, the 504 nm excitation maximum is due to the screening of carotene by xanthophylls, which decreases from 490 nm to longer wavelengths and leads to a corresponding increase in f, without reflecting

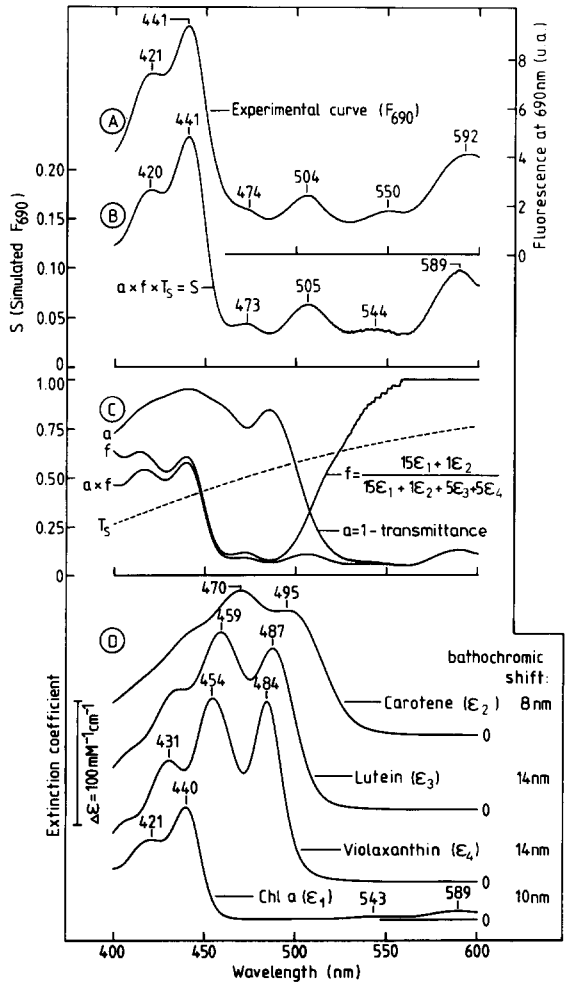

Fig. 3. Simulation of a fluorescence excitation spectrum of a primary leaf greened under intermittent light. (A) Excitation spectrum (F_{690}) of chlorophyll fluorescence at 690 nm of a primary leaf greened under intermittent light for 9 days. (B) Simulated fluorescence excitation spectrum $S(\lambda)$ calculated according to Eq. (12). Curve S is the product of T_s (scatter function), f (fraction of pigment absorbance associated with fluorescence), and a (total absorptance). (C) Spectra of T_s, f and a, the product of which yields curve S. Curve f was calculated according to Eq. (12) with bathochromically shifted absorbance spectra of isolated pigments (Fig. 3D) under the assumption that no energy transfer from xanthophylls to Chl a takes place and energy transfer from carotene to Chl a occurs with an efficiency of 100%. Total absorptance a ($=1-\text{transmittance} = 1-10^{-d\sum_i \varepsilon(\lambda)_i c_i}$) was calculated with the same pigment spectra. The scatter curve is $T_s = e^{-\lambda^{-4}r}$. Obviously the product of curve a and curve f already shows all extrema of the simulated excitation spectrum S. For the parameters see Table 1. (D) Normalised absorbance spectra of isolated pigments shifted to their putative in vivo position by the interval indicated (chlorophyll a: ε_1, carotene: ε_2, lutein ε_3, and violaxanthin ε_4).

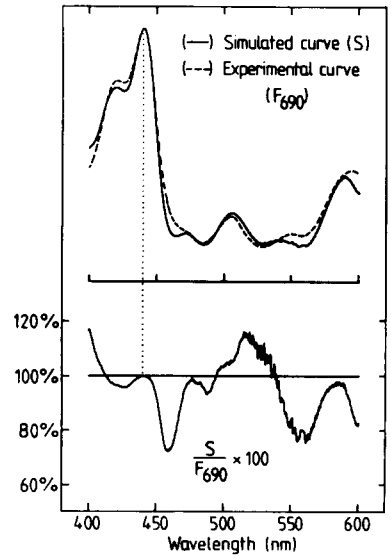

Fig. 4. Relative deviation of the simulated fluorescence excitation spectrum from its experimental counterpart. Spectra S (solid line) and F_{690} (broken line) are taken from Fig. 3. Previous to calculating the quotient S/F_{690}, the simulated spectrum was normalised to the main peak of F_{690}.

an absorbance maximum of any of the sample pigments.

At wavelengths shorter than 490 nm the fine structure of the simulated spectrum is mainly determined by f, whereas at wavelengths between 540 and 600 nm, where $f \approx 1$, it is solely dependent on the spectrum of a. Consequently, the simulated excitation bands at 420, 441, 550 and 589 nm originate from the corresponding absorbance maxima of Chl a, whereas the excitation band at 473 nm rather depends on the long-wavelength absorbance minimum of xanthophylls than on the carotene maximum at 470 nm (cf. Fig. 3).

According to our concept, light scattering of the leaf is considered to reduce the availability of excitation light for the leaf pigments. In contrast to this view, the primary effect of light scattering is often thought to be an increase of the optical path, thereby increasing the absorption by the sample pigments (for a review, see Schäfer et al. 1983). Since in most scattering media this increase in pigment absorption will tend to decrease with decreasing wavelengths (Butler 1964, 1972), qualitatively, an effect similar to our scattering correction may be expected. We are, how-

ever, well aware of the fact that the assumption of Rayleigh's law is a most simple hypothesis in the context of intact tissues, in which light scattering is though to be mostly Mie type (for a recent review see Vogelmann 1989). On the other hand, one has to adjust only one free parameter this way, whereas more complicated scattering functions would introduce more parameters and thus more arbitrariness.

On the whole, the simulated spectrum coincides well with its experimental counterpart (Fig. 4). A major deviation of the simulated spectrum from the experimental curve is observed at 460 nm and may be caused by a minor energy transfer from the xanthophylls to Chl *a* in vivo, since the main xanthophyll absorbance maxima are in this region. Deviations in the long-wavelength part may arise from a discrepancy of the long-wavelength Chl *a* maxima in vivo and the corresponding bands in $\varepsilon_1(\lambda)$.

The fitted parameter values are, on the whole, in good agreement with the values measured experimentally (Table 1), which supports the applicability of our simple model. The underestimation of the carotene concentration is most probably due to our assumption of complete energy transfer from carotene to Chl *a* and can be remedied (at the cost, however, of an additional parameter) by increasing the parameter c_2 to match the experimental carotenoid concentration whilst simultaneously lowering Φ_{T_2} in such a manner that the product of Φ_{T_2} and c_2 remains constant. Alterations of the simulated spectrum $S(\lambda)$ (Fig. 3) resulting from this procedure are below 5% (Pfündel 1989). The adaptation of c_2 yields a quantum yield $\Phi_{T_2} = 0.48$ which is more consistent with the energy transfer efficiencies published by Kramer et al. (1981) or by Wróbel and Hendrich (1989) (0.5–0.6 and 0.32–0.4, respectively) than with the efficiency of 1 given by Goedheer (1969).

We conclude that there are two principle ways in which the characteristic features of an excitation spectrum can arise. Firstly, all pigments may contribute to the process considered with equal quantum yields (as in Fig. 3, 550–600 nm); in this case, the excitation spectrum is a kind of absorptance spectrum. Secondly, the quantum yields may be different (as in Fig. 3, 400–550 nm); then the excitation spectrum results from a corrected absorptance spectrum and, in the extreme case, can be hardly traced back to the absorption properties of the active pigments. In both cases, scattering events are likely to distort the excitation spectrum as a whole.

Thus our model outlines, on the one hand, the uncertainties and limits of conclusions solely based on excitation spectra, and may serve, on the other hand, as a framework for an improved interpretation of fluorescence excitation spectra in particular, and for any kind of action spectrum in general.

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