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Comparison of the photoreduction of protochlorophyllide to chlorophyllide in leaves and cotyledons from dark-grown bean as a function of age

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Abstract

77 K fluorescence spectra of bean (*Phaseolus vulgaris* cv Commodore) leaves and cotyledons show the presence of active and inactive protochlorophyllides. The first detected product of the protochlorophyllide photoreduction is the chlorophyllide emitting fluorescence at 690 nm (C690) which is observed one day (leaves) and three days (cotyledons) after sowing. In cotyledons, C690 undergoes the 'rapid' and 'Shibata' shifts; in leaves, these spectral changes are age-dependent. In order to characterize the formation of C690, we have recorded 298 K fluorescence kinetics at 690 nm and the corresponding absorbance kinetics at 440 nm. The amplitude of the variations of both kinetics increases with the sample age. The absorbance and fluorescence kinetics can be modelized as a monoexponential law. The rate constant of the absorbance and fluorescence kinetics does not significantly change during the studied period (except for old cotyledons). The results presented in this paper give evidences for a low energy transfer between pigments during the photoreduction of protochlorophyllide at room temperature.

Abbreviations: Ax – absorbance intensity measured at x nm; Chlide – chlorophyll(ide); A.U. – arbitrary unit; Cx – chlorophyll(ide) fluorescing at x nm at 77 K; Fx – fluorescence intensity measured at x nm; PCR – NADPH:protochlorophyllide oxidoreductase (EC 1.6.99.1); Pchlide – protochlorophyllide; Px – protochlorophyllide fluorescing at x nm at 77K

Introduction

Several studies have been reported on the photoreduction of protochlorophyllide (Pchlide). Many of them have used old dark-grown (etiolated) leaves (15-day-old dicotyledons or 7-day-old monocotyledons) (Madsen 1963; Kahn and Nielsen 1974; Dujardin et al. 1990). Only few reports are devoted to the photoreduction of Pchlide in very young leaves (Klein and Schiff 1972; Schoefs et al. 1992a; Schoefs and Franck 1993).

Non-illuminated leaves contain two photoreducible Pchlides emitting fluorescence at 645 and 657 nm (P645 and P657) and at least two non-photoreducible Pchlides (P633 and P649) (Sironval et al. 1968a; Klein and Schiff 1972; El Hamouri and Sironval 1979; Böddi et al. 1992; Schoefs et al. 1992a; for a review, see Virgin 1981). P633 is regularly described as an enzyme-free pigment. It is now well accepted that photoactive Pchlides are ternary complexes between NADPH:protochlorophyllide oxidoreductase (PCR, EC 1.6.99.1), NADPH and P633 (Oliver and Griffiths 1982), whereas P649 is a ternary complex between PCR, NADP+ and P633 (El Hamouri and Sironval 1979; El Hamouri and Sironval 1980). Usually P645 does not emit fluorescence at 77 K because it transfers energy to P657 (Brouers et al. 1972; Dujardin 1975; Schoefs and Franck 1993).

Active Pchlides are phototransformed to the Chlide emitting fluorescence at 690 nm (C690) (Sironval et al.

1968a; Dobek et al. 1981; Schoefs and Franck 1993). Mathematical analysis of the kinetics of the light formation of C690 has especially been studied using old dark-grown leaves (Smith and Benitez 1954; Sironval et al. 1968a,b; Thorne 1971; Thorne and Boardman 1972; Nielsen and Kahn 1973) or their extracted Pchlide holochromes (Boardman 1962, 1966; Nielsen and Kahn 1973). The order of the kinetics is still a matter of debate and has been characterized either as a second order rate or as a sum of two first order ones or as a first order rate or neither first- nor second order rate (Smith and Benitez 1954; Boardman 1962, 1966; Sironval et al. 1968a,b; Nielsen and Kahn 1973). On the basis of these results, no firm conclusion concerning the mechanism of Pchlide photoreduction can thus be proposed. The Pchlide photoreduction has been illustrated by many absorbance and fluorescence kinetics (Brouers et al. 1972; Thorne and Boardman 1972; Vaughan and Sauer 1974) the difference in the rate constants between these absorbance and fluorescence kinetics leads to the conclusion of an energy transfer between active Pchlides and C690 during its formation (Thorne and Boardman 1972; Brouers and Sironval 1974; Vaughan and Sauer 1974). From these results, it was concluded that active Pchlides consist in aggregates of ternary complexes of different sizes (Kahn et al. 1970; Brouers et al. 1972; Sironval and Kuyper 1972; Thorne and Boardman 1972; Vaughan and Sauer 1974; Brouers and Sironval 1978; Sironval and Brouers 1980). Recent circular dichroic studies of in vitro protochlorophyllide forms have confirmed the aggregated state of the pigments (Böddi 1990).

As leaves, cotyledons contain active and inactive Pchlides (Goedheer and Verhülsdonk 1970; Rebeiz et al. 1970; El Hamouri and Sironval 1979) and become green in the light. To our knowledge, there is only one report on the active Pchlide photoconversion in cotyledons (*Pharbitis nil*, Ogawa and Konishi 1979). These authors found a second order law for the kinetic.

In this report we have examined how chlorophyll formation occurs in bean leaves and cotyledons as a function of their age, by means of 77 K fluorescence spectra. The question of the reaction order of the phototransformation of active Pchlides has been reinvestigated by computer analysis of room temperature absorbance and fluorescence kinetics. The results are discussed in terms of pigment-protein associations and of energy transfer between pigments.

Materials and methods

Culture

After 16 h of imbibition in air enriched tap water, *Phaseolus vulgaris* L. cv Commodore seeds were grown on tap water in darkness at 298 K. (Schoefs et al. 1992b) for different periods of one to 14 days. Leaves and cotyledons were harvested in the dark at every day of growth using a weak green light. The seedling age is expressed in days after sowing. The morphological state of dark-grown bean seedlings was described by Khandakar and Bradbeer (1988) and by Schoefs and Franck (1993). Seedlings were dissected in darkness before experiments.

440 nm absorbance and 690 nm fluorescence kinetics

Light-induced variations of absorbance at 440 nm and fluorescence at 690 nm were recorded at 298 K using the device described in Schoefs et al. (1993). Briefly, the phototransformation of Pchlides was triggered using a 632.8 nm exciting light from a He-Ne laser (Spectra Physics, 5 mW cm^{-2}). The analytical light (440 nm) was selected using a monochromator. In front of each photomultiplier tube (EMI type 9558, S20), a blue filter (440 nm, bandwidth: 36 nm) or a red filter (690 nm, bandwidth: 30 nm) is placed in order to select the fluorescence of the transmitted light. The absorbance and fluorescence signal were recorded on a rapid paper recorder (Gould, Brush 220).

Light-induced variations of fluorescence at 690 nm were also recorded at 273 K using the same device. For this measurement, the sample was immersed in a melting ice bath. As for spectra, each kinetic was realized with a sample surface of 22 mm^2 which could be covered either by one cotyledon or a variable number of leaves. The samples were stuck on the section of an optic guide with a non-fluorescent adhesive paper.

Computer analysis and calculation

Three absorbance kinetics and ten fluorescence kinetics were recorded on average. Experimental points (around 25 s^{-1}), deduced from each kinetic curve, were analysed in a two-step procedure. Firstly, a computer program (Garnir and Monjoie 1981) was used to adjust each curve with a parametric function of the form:

$$F(t) = a(1 - e^{-kt})$$

For each curve the best value of 'a' and 'k' have been estimated by least squares fitting techniques. The 'a' parameter has been used to normalize every experimental curve by dividing each amplitude by the estimated value of the 'a' parameter and all the normalized curve obtained in the same conditions have been summed. Secondly, these resulting normalized kinetic curves have been subsequently fitted by another computer program (Monjoie and Garnir 1993) in order to discriminate between mono or multi exponential behaviour. Again, the same least-squares fitting procedure was used to adjust a function of the form:

$$F(t) = 1 - \sum_{i}^{n} a_n e^{-k_n t} \text{ with the constraint that } \sum_{i}^{n} a_n = 1$$

Different values of the reaction order 'n' were tried and for each curve we monitored the decrease in the reduced X^2 as the value of 'n' increased. However, no firm conclusion can be drawn from this analysis. No striking evidence for a multiexponential behaviour could be assessed and all the curves could fit very well with a monoexponential law (n = 1). It can be noted that if a multiexponential behaviour is expected and that the components cannot be distinguished from one another, 'n' only represents the apparent reaction order. We have considered that a value for the rate constant (k) was aberrant when $k_{(t)} - k_{(t-1)} > E/3$ where $E = k_{Max} - k_{Min}.k_{(t)}$ represents the value of the rate constant for the age t whereas k_{Max} and k_{Min} represent the maximal and the minial values found for the constant rate (Rorabacher 1991).

77 K fluorescence spectra

Non-illuminated samples were frozen in liquid nitrogen, either before or immediately after a 1 ms white flash (Multiblitz, 125 J) or after a subsequent dark period (0–30 minutes). The time required to freeze the samples has been evaluated at two or three seconds (data not shown). 77 K fluorescence spectra were recorded with the apparatus described by Sironval et al. (1968b). The surface of exposed sample was 22 mm^2 which could be covered either by one cotyledon or a variable number of leaves (1 to 8) depending on their age. Due to the low amounts of pigments in the young samples, high amplification was necessary for fluorescence detection. For this reason it was not possible to avoid a drift in the baseline, which did not in general markedly affect the position of the peak maxima, as verified in some control experiments (data not shown). The spectra were neither normalized nor corrected for detector sensitivity.

77 K absorbance spectra.

The spectra were recorded using an Optical Multichannel Analyser (OMA II, Princeton Instruments). The analytical light was a projector lamp (Sylvania 21 V, type DKM). The wavelength detector resolution was 0.5 nm. In order to record the spectra at definite moment of the photoreduction of active Pchlide, different samples were irradiated in the same conditions as those used for kinetic measurements (see above). The rapid fall of the sample in liquid nitrogen was optimized by an electromagnetic home-made device (the time of the travel is less than 0.1 s).

Results

77 K fluorescence spectra

Leaves

Figure 1A shows 77 K fluorescence spectra of non-illuminated leaves of different ages. Whatever the age, the spectrum presents emission bands at 630 and 657 nm corresponding to inactive and active Pchlide respectively (P633 and P657; Sironval et al. 1968a,b). The relative amplitude of the 630 nm band decreases while the 657 nm band increases during the dark-growth. The shoulder around 675 nm could correspond to an inactive Pchlide (Böddi et al. 1992) or Chlide (Durchan et al. 1993). A weak fluorescence band around 715 nm is observed.

Figure 1B–D presents 77 K fluorescence spectra of flashed leaves of different ages maintained in the dark for different time periods. Whatever the leaf age, the flash induces a dramatic decrease in the 657 nm band and the appearance of a new band at 690 nm traducing the formation of C690 (Fig. 1B). A band around 675 nm is also observed. It results mainly from the quick transformation of C690 to C675 (Schoefs and Franck 1993). The younger the samples, the higher the relative intensity at 675 nm. When flashed leaves are placed in the dark (for 1 s to 30 min) before freezing, spectral shifts are observed: the shift from 690 to 696 nm (called the 'rapid' shift; Fig. 1C curves b–c) and the one from 696 to 675 nm (called the Shibata shift; Shibata 1957;

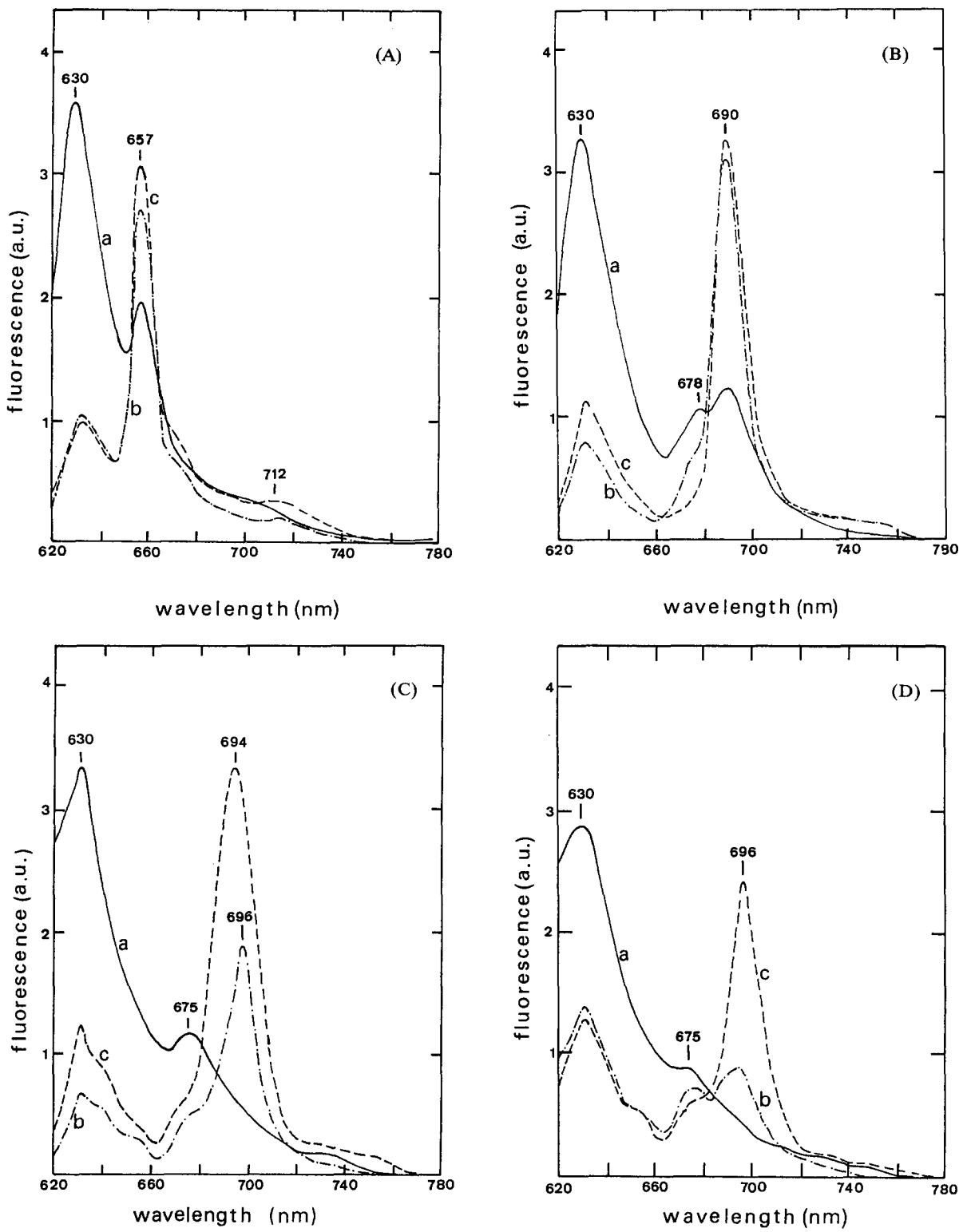

Fig. 1. 77 K fluorescence spectra of bean leaves. (A) non-illuminated, (B) frozen immediately after the 1 ms flash, (C) frozen 60 s after the flash and (D) frozen 300 s after the flash. Ages: 2 days (a), 8 days (b) and 15 days (c).

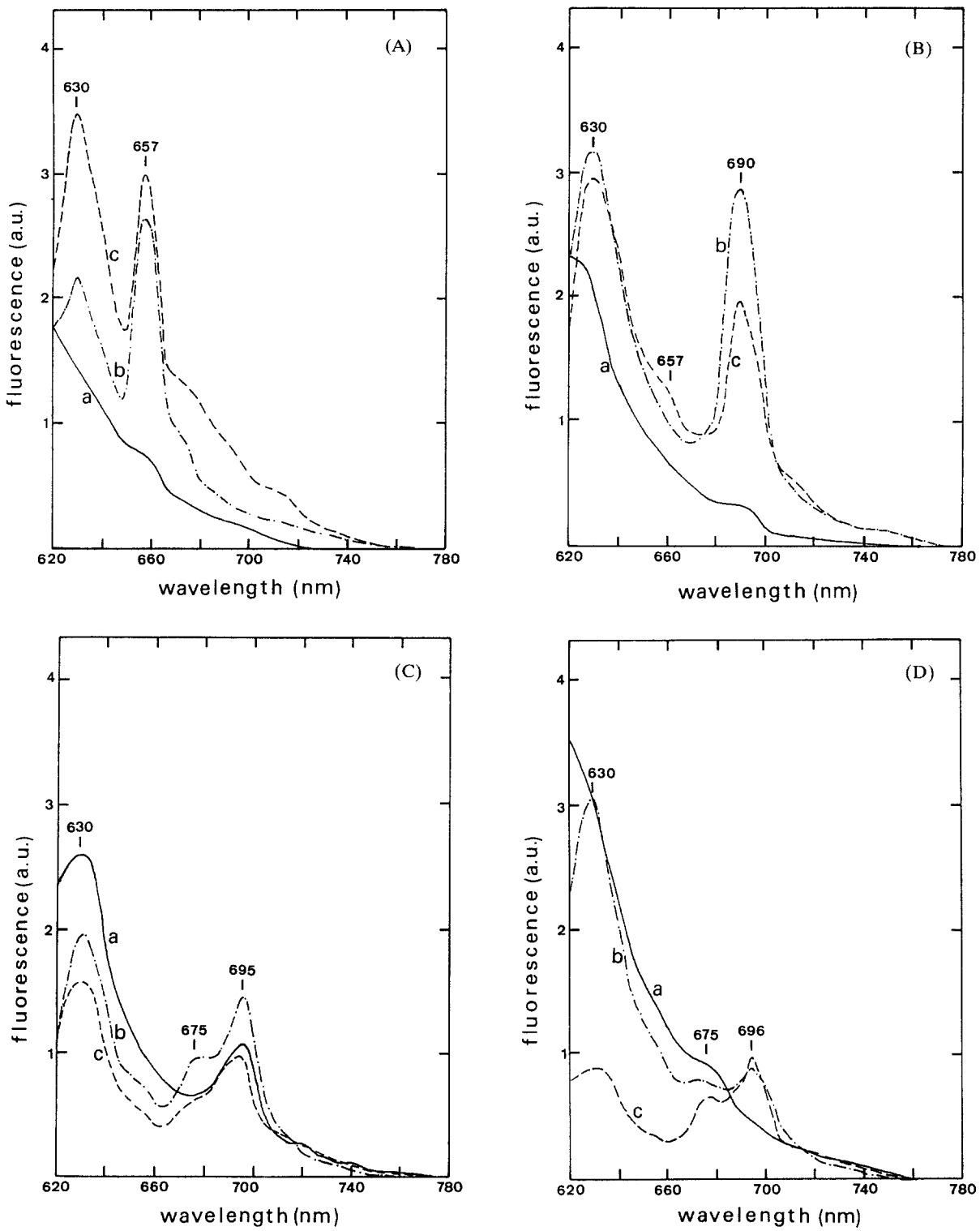

Fig. 2. 77 K fluorescence spectra of bean cotyledons. (A) non-illuminated, (B) frozen immediately after the 1 ms flash, (C) frozen 60 s after the flash and (D) frozen 300 s after the flash. Ages: 2 days (a), 8 days (b) and 15 days (c).

Thorne and Boardman 1972) (Fig. 1D curves b, c). Only a small part of Chlide formed in young leaves performs the two shifts. The main part directly shifts to 675 nm (Fig. 1C–D, curve a). Interestingly both active Pchlide forms are observed in 77 K fluoresence spectra of flashed leaves placed in darkness (Fig. 1C–D).

Cotyledons

Figure 2A shows 77 K fluorescence spectra of cotyledons from dark-grown plants of different ages. According to Schoefs and Franck (1990), no pigment fluorescence is detected before the 3rd day of growth. Active Pchlide appears at that time. In young cotyledons, the 77 K fluorescence at 657 nm is only observed (curve a). In older samples, emission bands at 630 and 657 nm are clearly detected (Fig. 2A, curves b–c). As for leaves, shoulders at 675 and 715 nm are observed (Fig. 2A, curve c).

The white flash induces the disappearance of the band at 657 nm (P657) and the appearance of a new band at 690 nm (C690, Fig. 2B). Occasionally a weak shoulder around 675 nm is still observed (data not shown). When flashed cotyledons are placed in darkness before freezing, the 'rapid' shift occurs (Fig. 2C). In contrast to leaves, the fluorescence band at 696 nm is always the main one found after a short dark-period (Fig. 2C). The Chlide fluorescence band then shifts to 675 nm (Fig. 2D). The younger the sample, the quicker the shift.

440 nm absorbance and 690 nm fluorescence kinetics

Figure 3 shows an example of crude 440 nm absorbance and 690 nm fluorescence kinetics of the photoreduction of active Pchlides at 298 K of 7-day-old bean leaves. The kinetics relative to cotyledons have the same shape (data not shown). The 440 nm absorbance was chosen because it is the Soret absorbance maximum of Pchlide and Chlide which does not undergo spectral shifts after Pchlide reduction (Shibata 1957; Bertrand et al. 1988; Dujardin et al. 1990). The 440 nm absorbance intensity decreases (Bereza and Dujardin 1981; Dujardin et al. 1990) till a plateau. The lower molar extinction coefficient of Pchlide at 440 nm is responsible for this decrease (French 1960).

After an initial rise corresponding to the shutter aperture (1.5 ms), the 690 nm fluorescence intensity increases until a maximum 'B' due to the formation of C690. Then it decreases to a minimum value 'S' due to

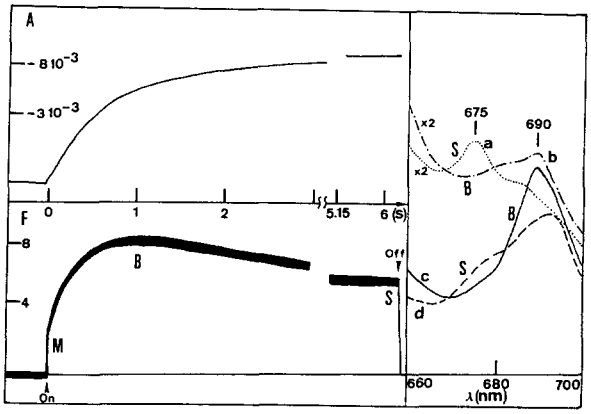

Fig. 3. Room temperature 440 nm absorbance (A) and 690 nm fluorescence (F) kinetics of 7-day-old bean leaves. The insert shows the 77 K fluorescence spectra of young leaves (a, b) and old leaves (c,d) when the fluorescence kinetic rises the 'B' and 'S' points.

the transformation of C690 to C675 and/or C696. The spectra of Fig. 3 show the predominance of C690 when the fluorescence kinetics reach the maximum 'B' (Fig. 3, spectra b, c) and the predominance of C696 when the fluorescence kinetics reach the minimum 'S' in old leaves but the predominance of C675 is observed in young leaves (Fig. 3, spectra a, d). Corresponding spectra for cotyledons are similar to those presented for old leaves (data not shown). In the 273 K fluorescence kinetics, the 'M -> B' transition is followed by a plateau indicating that the rapid shift is inhibited at this temperature (data not shown).

Computer analysis of the photoreduction kinetics ('M -> B' transition)

Figure 4 shows room temperature monoexponential normalized and modelized 440 nm absorbance kinetics and 690 fluorescence kinetics of leaves and cotyledons of different ages. No significant difference due to age is observed, either with leaves or with cotyledons except for the fluorescence kinetic of old cotyledons (Fig. 4B). A good fit has been obtained at all ages and at both temperatures using monoexponential equations.

Figure 5 shows the evolution of the modelized amplitude of the room temperature 440 nm absorbance and the 690 nm fluorescence kinetics as a function of the leaf or cotyledon age. During the studied period, the amplitude of the absorbance kinetic varies sigmoidally up to 10 days and then decreases. The amplitude of the fluorescence kinetics seems to increase more linearly.

Figures 6A and B present the evolution of the rate constant of the room temperature fluorescence kinetics

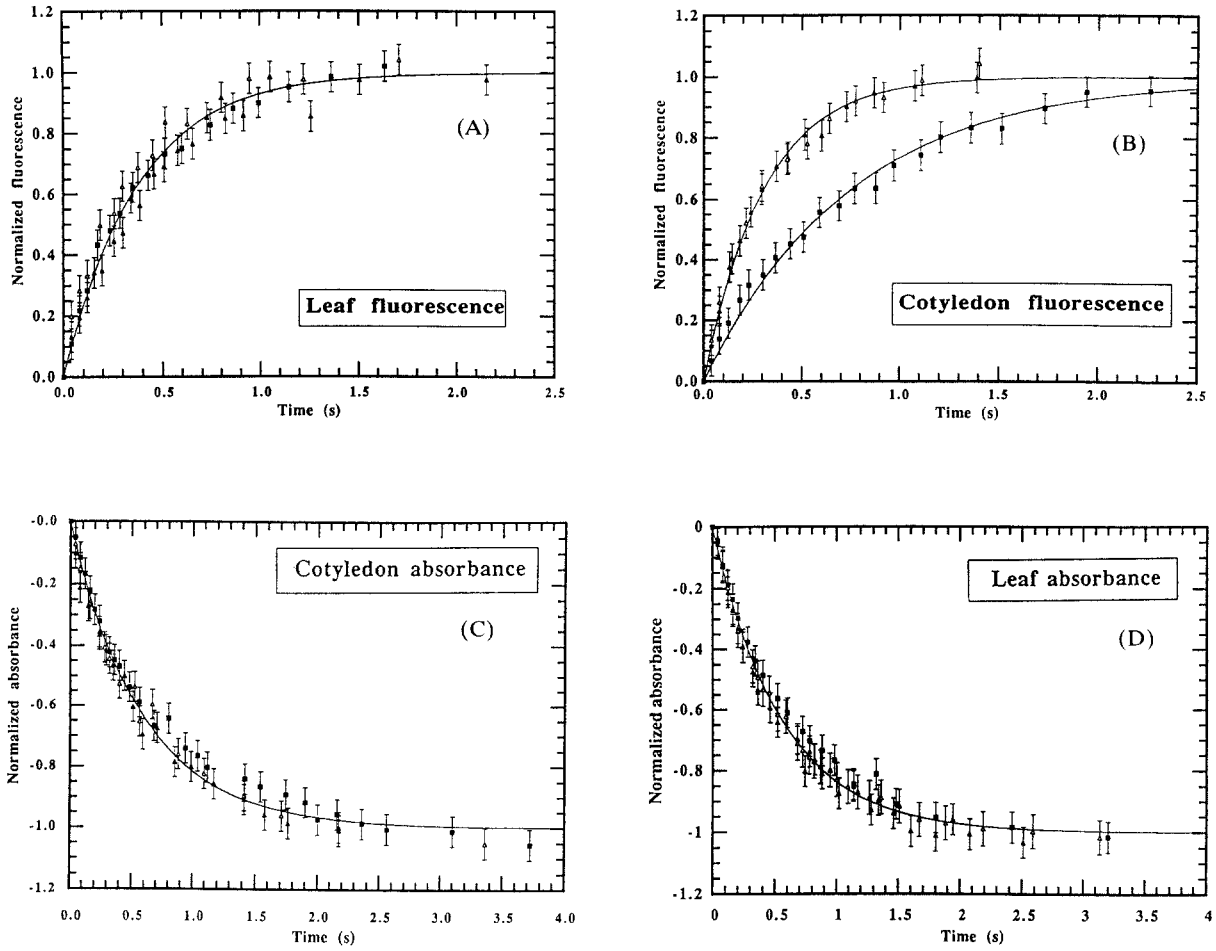

Fig. 4. Room temperature normalized 690 nm fluorescence (A, B) and 440 nm absorbance (C, D) kinetics of the photoreduction of active Pchlide to C690 in leaves (A, D) and in cotyledons (B, C). Ages: 3 days (solid triangles); 5 days (open triangles) and 10 days (solid squares). Modelized 5-day-old fluorescence and absorbance kinetics are represented by the solid line. Modelized 10-day-old cotyledon fluorescence kinetic is also presented (B).

of leaves and cotyledons. For leaves, this rate constant remains equal to about 2.3 s^{-1}. The rate constant of the cotyledon fluorescence kinetics decreases with age from 3.5 to 2.3 s^{-1}.

Figures 6C and D exhibit the evolution of the rate constant of the normalized and modelized absorbance kinetics during the first 14 days of growth. It appears that the rate constant of those kinetics remains around 1.6 s^{-1} for cotyledons and around 2.0 s^{-1} for leaves. The data for the youngest leaves (1–2 days old) are not close to 2.0 s^{-1} owing to the difficulty in accurate estimation of the very small amplitude of the kinetics.

The rate constant of the 273 K fluorescence kinetics are reported in Table 1. Comparing these data and the ones plotted in Fig. 4A and B, we see that the rate constant at 273 K is higher than the one at 298 K. This increase was already observed by Sironval et al. (1968 a, b), Sironval and Brouers (1970) and Ogawa and Konishi (1979).

Table 1. Rate constants of the 273 K fluorescence kinetics as a function of leaf and cotyledon age

Age (days)	Rate constants (s-1)	
	Leaf	Cotyledon
2	4.6	20.0
3	2.9	3.3
4	3.0	3.2
5	3.9	3.3
7	3.0	3.3

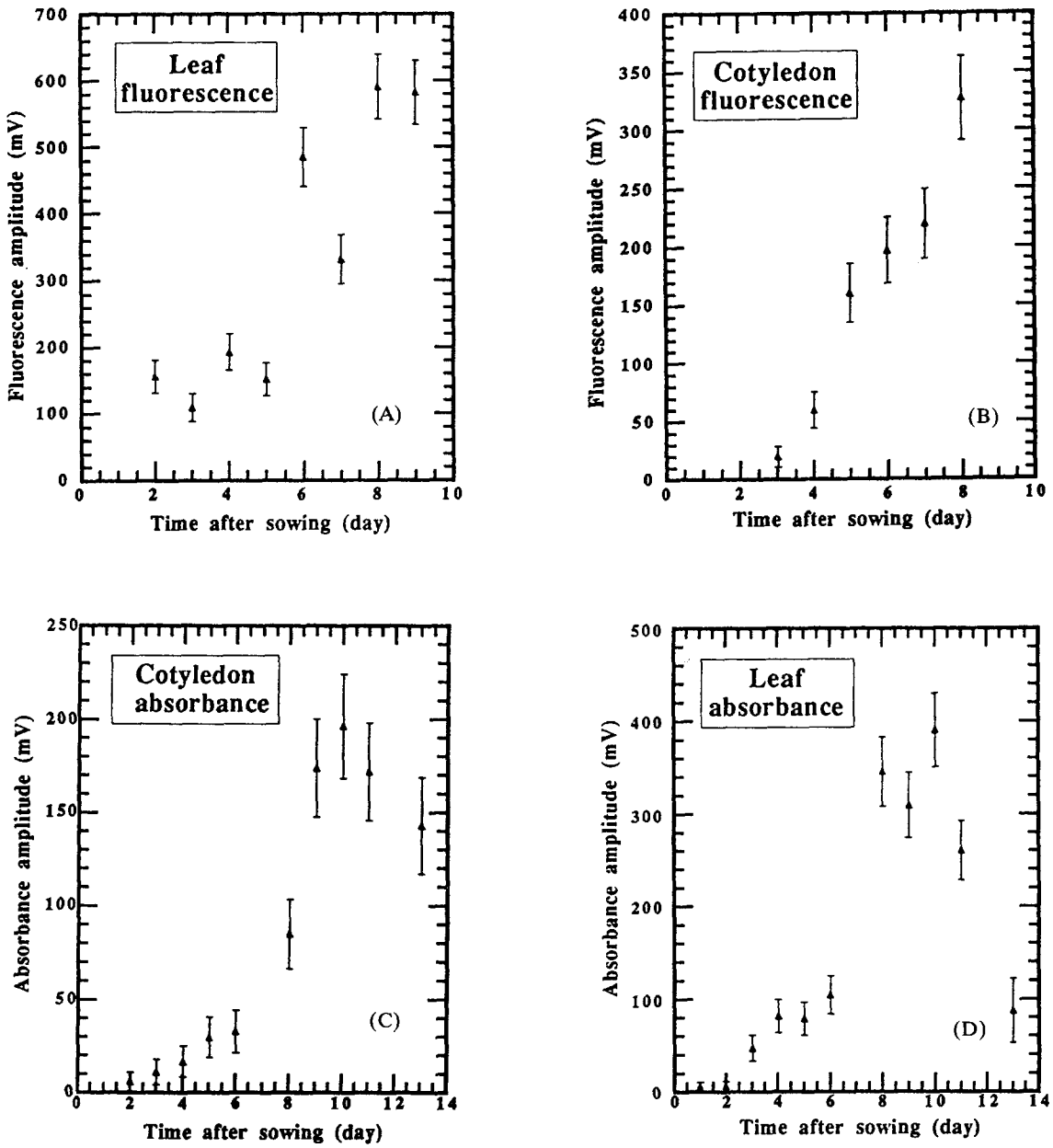

Fig. 5. Evolution of the amplitude of the modelized fluorescence kinetics at 690 nm (A, B) and modelized absorbance kinetics at 440 nm (C, D) of leaves (A, D) and of cotyledons (B, C) as a function of age.

We also recorded absorbance difference spectra (light minus dark) at different illumination times (Fig. 7). For practical reasons, this experiment was only performed with 10-day-old leaves.

The spectrum of one non-illuminated leaf always shows the well-known band at 650 nm and a shoulder around 638 nm (data not shown). These two absorbance maxima are usually attributed to P645 and P657 (Kahn et al. 1970; Brouers et al. 1972). Upon illumination, both absorbance bands decreased whereas a new band at 678 nm, corresponding to C690, progressively increases. This is illustrated by the difference spectra presented in Fig. 7). The comparison of the difference spectra at increasing illumination times shows that the two Pchlide absorbance bands decrease together. No significant difference in the relative decrease

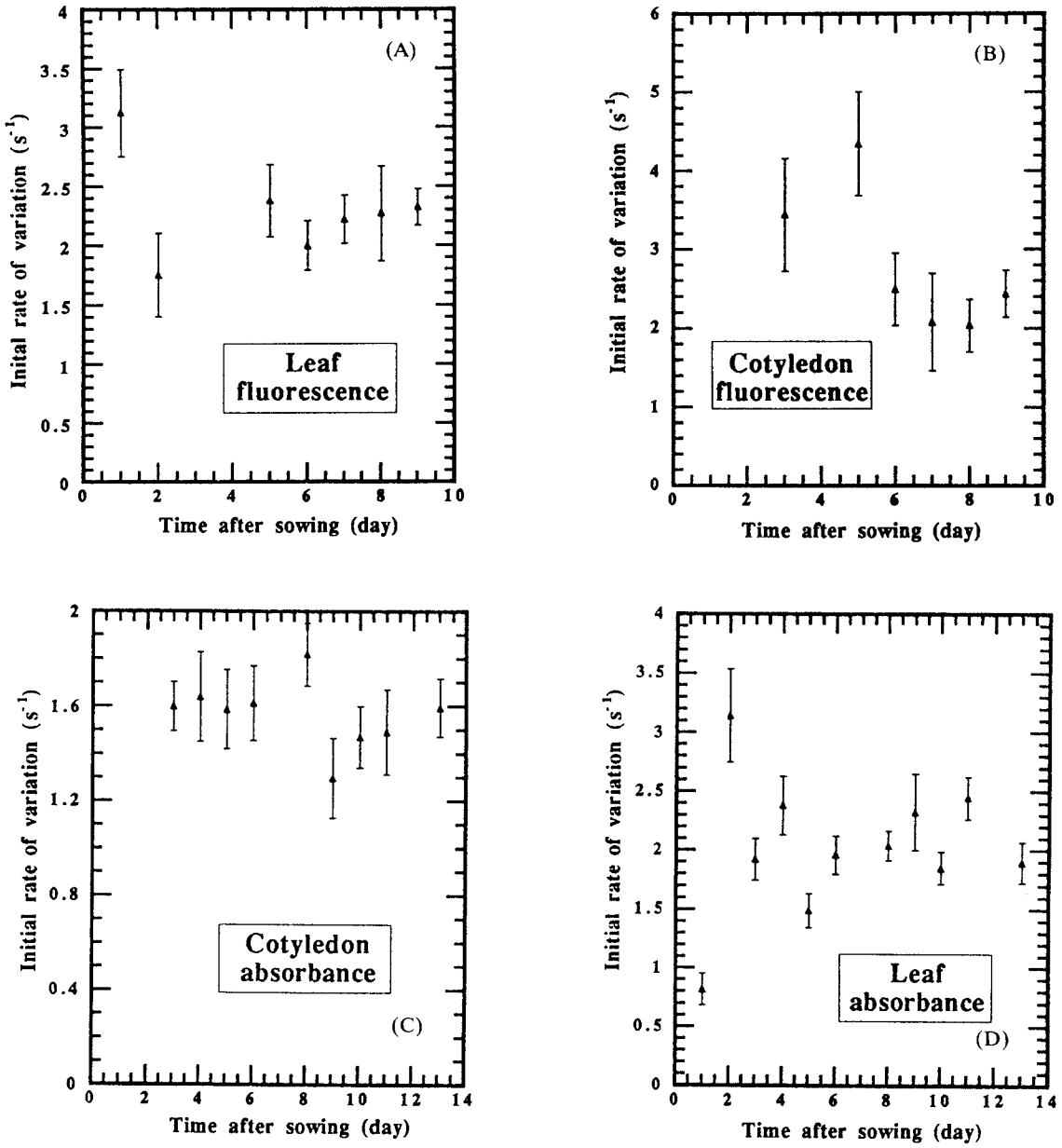

Fig. 6. Evolution of the rate constant of the modelized fluorescence kinetics at 690 nm (A, B) and modelized absorbance kinetics at 440 nm (C, D) of leaves (A, D) and of cotyledons (B, C) as a function of age.

rate of the two components (638 and 650 nm) can be observed.

Discussion

One day after sowing, P633 and P657 appear in non-illuminated leaves (absorbance maximum at 628 nm and 650 nm respectively, Fig. 7; Kahn et al. 1970; Brouers et al. 1972). These Pchlide forms appear in 3-day-old non-illuminated cotyledons. In *Cucumber* cotyledons, Pchlide is already detected one day after sowing (Rebeiz et al. 1970). The weak band around 710 nm observed in spectra of non-illuminated samples is due to a vibrational band of P657 (Sironval et al. 1968b; Litvin and Stadnitshuk 1980; Böddi

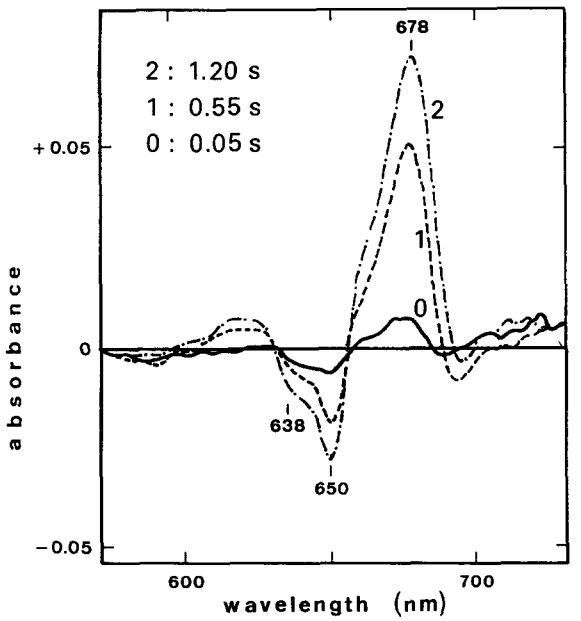

Fig. 7. 77 K difference absorbance spectra of different 10-day old leaves illuminated at room temperature for indicated times. The reference spectrum was always the non-illuminated leaf.

et al. 1992). Although P645 has been detected by absorbance measurement (Fig. 7; Sironval et al. 1968a; Klein and Schiff 1972), this active Pchlide has never been observed in the 77 K fluorescence spectra of non-illuminated samples. This observation can only be explained by a transfer of energy from P645 to P657.

Both continuous light and flashes trigger the transformation of active Pchlide to C690 (Fig. 1B, 2B, 3 left part, Fig. 7) which is considered as the first product of the phototransformation [here we do not take into account the long-wavelength non-fluorescing pigment forms which are known to be formed before C690 (Dujardin and Correia 1979; Inoue et al. 1981; van Bochove et al. 1984)]. C690 has its absorbance maximum at 678 nm (Fig. 7; Kahn et al. 1970; Brouers et al. 1972).

As we have recently reported, the fate of C690 is different in young and in old leaves (Schoefs et al. 1992a; Schoefs and Franck 1993). In 2-day-old leaves, the major part of C690 is directly transformed to C675 while the remainder undergoes the so-called 'rapid' shift to form C696. The proportion of Chlide molecules undergoing the 'rapid' shift increases with leaf age. In 15-day-old samples, C690 mainly exhibits the 'rapid' shift. In this case, the fluorescence of C675 only appears as a shoulder (Sironval et al. 1968a; Thorne 1971; Thorne and Boardman 1972). Whatever their age, the cotyledons behave as old leaves (compare Figs. 1B, C and 2B, C). The lifetime of C696 seems to increase with the cotyledon age.

In leaves, C696 probably consists of Chlide-PCR-NADPH complexes synthesized when prolamellar bodies are well developed (El Hamouri and Sironval 1979; Oliver and Griffiths 1982). It is known that, at room temperature, C696 is a quencher of chlorophyll(ide) fluorescence (Jouy and Sironval 1979), indicating that C696 is actually an aggregate of pigment-protein complexes. Because C675 is not quenched by C696, we proposed that it corresponds to free Chlide (Schoefs and Franck 1993). This Chlide spectral form is particularly abundant when prolamellar bodies are absent (Klein and Schiff 1972; Schoefs and Franck 1993). The occurrence of C696 in fluorescence spectra of young leaves indicates the existence of some environmental conditions allowing aggregation of pigment-protein complexes. Hence we can assume that spectral forms and ultrastructural features are related. Thus it is not surprising that the formation of C696 is always found predominant in cotyledons since prolamellar bodies were observed in their non-illuminated plastids (Huber and Newman 1976) and even in dry seeds (Webster and Leopold 1977; Cachon and Geneves 1985).

The 690 nm fluorescence kinetics of both cotyledons and leaves present two phases (Fig. 3). The initial fluorescence, after shutter aperture (M) is probably due to the weak fluorescence emitted by the non-photoreducible Pchlide (Böddi et al. 1992) and/or by the small pool of Chlide formed in darkness (Adamson et al. 1990; Durchan et al. 1993); the 'M -> B' phase corresponds to the formation of C690 (Sironval et al. 1968a,b, 1984). The 'B -> S' phase is due to the transformation of C690 to other Chlide spectral forms. In old leaves, the decrease signifies the predominant transformation of C690 to C696 which quenches the room temperature fluorescence of other transferring pigments, and has a very low fluorescence yield at room temperature (Jouy and Sironval 1979; Jouy 1982). In young leaves, C690 is mainly transformed into C675 (Fig. 3 insert; Schoefs and Franck 1993).

The absorbance kinetics at 440 nm only present a rapid decrease reflecting the phototransformation of Pchlide to Chlide. This phase is followed by a plateau indicating the absence of any pigment destruction (Fig. 3 left part). The decrease in the amplitude of the absorbance kinetics and the increase in the amplitude of the fluorescence kinetics with sample age demonstrate the enlargement of the Pchlide pool (Fig. 3A). This is also supported by the increase in the 657 nm

band and by the decrease in the 633 nm one in the 77 K fluorescence spectra. Interestingly, an increase in the barley leaf PCR content during dark-growth has been recently reported (Savchenko et al. 1990). However, the PCR accumulation curve is not the same as the increase in the 440 nm amplitude reported here.

The rate of the transformation of active Pchlide to C690 is dependent on the temperature and on the actinic light intensity (Smith and Benitez 1954; Sironval and Brouers 1970; Goedheer and Verhülsdonk 1970). Consequently, in this report, we have recorded the kinetics for a short period (a few seconds), at room temperature and using an exciting beam strong enough to complete the reaction within 4 seconds in order to avoid Pchlide resynthesis during the measurements and to limit to a minimum the interaction between the formation of C690 and its transformation to C696.

Both room temperature normalized absorbance and fluorescence kinetics fit very well with a monoexponential law (Fig. 4). The rate constant of the 440 nm absorbance kinetics remains approximately the same during the whole growth of leaves and cotyledons, showing that the phototransformation occurs in the same way whatever the age of the seedlings. Our statistical analysis of the data does not give evidence for more than one component indicating that a possible second component would have either a very low amplitude or approximately the same rate. The kinetics of photoreduction was previously reported as a second order reaction or as a sum of two first order reactions (Boardman 1962; Sironval et al. 1968b; Thorne and Boardman 1972; Ogawa and Konishi 1979). Quite different rates for the two exponentials have been found by Boardman (1962). The differences between our results and those of Smith and Benitez (1954); Virgin (1955); Boardman (1962); Boardman (1966); Sironval et al. (1968a,b); Thorne and Boardman (1972); Nielsen and Kahn (1973); Vaughan and Sauer (1974), can be explained as follows: some of these authors have performed kinetics for a long period (150 s to 30 min) during which active Pchlide can be resynthesized even in the light (Gassman and Bogorad 1967; Shlyk et al. 1969; Granick and Gassman 1970; Schoefs and Franck 1990; Franck and Strzalka 1992). This Pchlide is of course immediately photoreduced to Chlide which influences the shape of the kinetic. Other authors have reconstituted the kinetics from spectra recorded at low temperature (77 K). At such a low temperature, the chlorophyll(ide) fluorescence is magnified (Harnischfeger 1977; Huner et al. 1992). Unfortunately, the fluorescence yield of the different spectral forms of pigments (P657 and C690) does not increase similarly with the temperature (Goedheer and Verhülsdonk 1970). These facts together modify the kinetic pictures. According to Kahn et al. (1970) and Thorne (1971), 'the heigth of the fluorescence emission band at 655 nm at 77 K may not be used to estimate the amount of photoconversion because of energy transfer to Chlide *a*', also influencing the shape of the kinetics.

In order to eliminate the low contribution of C696 in the room temperature fluorescence kinetics, we have recorded the variation of the kinetics at 273 K. At this temperature, the formation of C696 is inhibited. The modelization of those kinetics also shows a monoexponential behaviour. This confirms the monoexponential character of the room temperature kinetics.

It is generally accepted that active Pchlide occurs in vivo as two aggregates of protein-Pchlide complexes (Sironval et al. 1968a; Sironval et al. 1968b; Böddi 1990). However the possibility that both absorbance maxima arise from the same pigment-protein complexes or of two states in dynamic equilibrium has also been proposed (Kahn and Nielsen 1974). In the first hypothesis (two independent forms correponding to the 638 and 650 nm absorbance bands), the two forms should be transformed at a rate which would depend on their respective excitation coefficient at the excitation wavelength. Our findings of monoexponential kinetics and of simultaneous decrease in the two absorbance bands under 632.8 nm excitation at room temperature are not in favour of this hypothesis; unless excitation energy transfer between the two forms exactly compensates the difference in direct excitation. By exciting preferentially the 650 nm form using 671 nm light, a condition which practically eliminates the possibility of energy transfer, Kahn and Nielsen (1974) reached the same conclusions. On the other hand, the existence of only one physical state is not favoured in view of the progressive increase in the 650 versus the 638 nm band during dark-growth (Klein and Schiff 1972). More investigation is required to test further the possibility of the two forms in a dynamic equilibrium.

In leaves, the rate constant of the absorbance and fluorescence kinetics remains similar to the age excepted for the very young samples (see the 'Materials and methods' section). From those results, we conclude that the energy transfer from Pchlide to C690 occurs with a low efficiency at room temperature. In cotyledons, the same conclusion can also hold. However in that case, the energy transfer slightly increases with the age. The weak efficiency of excitation energy transfer at physiological temperature is in line with the high

efficiency of Pchlide photoreduction and the low fluorescence yield of active Pchlides at room temperature.

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