

## Influence of Kinetin on Protochlorophyll(ide) Accumulation and on the Shibata Shift in *Raphanus* Seedlings

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**Abstract.** Radish seedlings (*Raphanus sativus* L. Saxa Treib) were grown in the dark with or without added kinetin (2 mg/l = 9.29  $\mu$ M). Low-temperature (77° K) fluorescence emission and absorption spectra of etiolated cotyledons were registered at increasing seedling age before and immediately, 30 s and 30 min after one 1-ms flash. Kinetin was found to induce a higher accumulation of the phototransformable protochlorophyll(ide) P<sub>657-650</sub> in the etiolated cotyledons, especially from day 6 to day 10 after germination. The amount of the P<sub>657-650</sub> protochlorophyll(ide) resynthesized during a 30-min dark period after a 1-ms flash decreased with seedling age. It was smaller in cotyledons from kinetin-treated seedlings at day 6 after germination and at that age only. The ability to perform the Shibata shift decreased with increasing seedling age. In cotyledons from 10- and 13-day-old seedlings, the shift was accomplished to a greater extent when the plants were grown in the presence of kinetin.

**Key words:** Cytokinin – Protochlorophyll – *Raphanus* – Shibata shift.

### Introduction

Kinetin, a synthetic cytokinin, is known for its influence on protein synthesis and cell division (review: Skoog and Armstrong, 1970; Hall, 1973). One of the main sites of action of cytokinins is the chloroplast (Kull, 1972). Cytokinins promote the differentiation of plastids (Stetler and Laetsch, 1965; Harvey et al., 1974; Woźny and Szweykowska, 1975) and increase the number of chloroplasts per cell (Laetsch and Boasson, 1972). The photosynthetic activity rises (increased CO<sub>2</sub> fixation: Wareing et al., 1968; Adedipe et al., 1971; higher activity of Calvin cycle enzymes:

Harvey et al., 1974; higher Hill activity: Buschmann and Lichtenthaler, unpublished; and increased photophosphorylation: Yakushkina and Pushkina, 1971, 1975). Inside the chloroplasts cytokinins promote the accumulation of several components such as chlorophyll (Sugiura, 1963; Banerji and Laloraya, 1967; Fletcher and McCullagh, 1971 a, b; Yakushkina and Pushkina, 1971; Fletcher et al., 1973; Straub and Lichtenthaler, 1973; Woźny and Szweykowska, 1975), plastoquinone-9 (Straub and Lichtenthaler, 1973), and P<sub>700</sub> (Buschmann and Lichtenthaler, 1977).

The influence of cytokinins on the synthesis of protochlorophyll(ide) was investigated in excised leaves. An increase of the accumulation in the light (Banerji and Laloraya, 1967) and of the resynthesis during a 24-h dark phase (Shlyk and Averina, 1969; Shlyk et al., 1970) was reported. According to Fletcher (Fletcher and McCullagh, 1971 a; Fletcher et al., 1973) cytokinins increase the synthesis of  $\delta$ -aminolevulinic acid by stimulating  $\delta$ -aminolevulinic acid synthetase in the presence of light.

We investigated the influence of kinetin on the first steps of chlorophyll biosynthesis in etiolated radish cotyledons. Fluorescence and absorption spectra of intact leaves were chosen as a tool for that purpose because they provide information on the early accumulation of pigments and on their binding to proteins (Shibata, 1957; Goedheer, 1967; Sironval et al., 1968).

### Materials and Methods

Radish seedlings (*Raphanus sativus* L. Saxa Treib) were grown in complete darkness in vermiculite moistened with tap water. Added kinetin was present in the water at a concentration of 2 ppm (9.29  $\mu$ M). All manipulations were carried out with a green safe light (maximal transmission: 540 nm).

Cotyledons were harvested from 4, 6, 8, 10, and 13-day-old seedlings and placed in holders (Sironval et al., 1968). The samples

were frozen to 77°K in liquid nitrogen before, immediately, 30 s, or 30 min after having received one flash (a 1-ms polychromatic flash, Multiblitz Report Proba, electric energy 125 J, color temperature 5800°K).

The low temperature absorption spectra were registered using a CARY 17 R spectrophotometer (for details: Dujardin, 1976). The fluorescence emission spectra were registered using an apparatus described previously (Sironval et al., 1968: photomultiplier: EMI 9558 B, response S20, excitation light: 436 nm). The values used for calculating the ratios in Figures 3, 6, and 7 have been corrected for the sensitivity curve of the photomultiplier (fluorescence) or for the baseline change (absorption). The spectra are reproduced without any further correction.

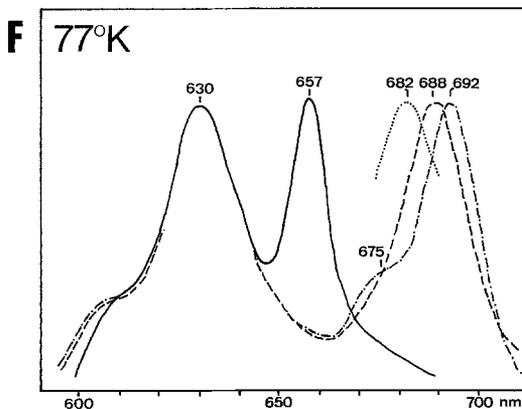
## Results

### 1. Etiolated Cotyledons

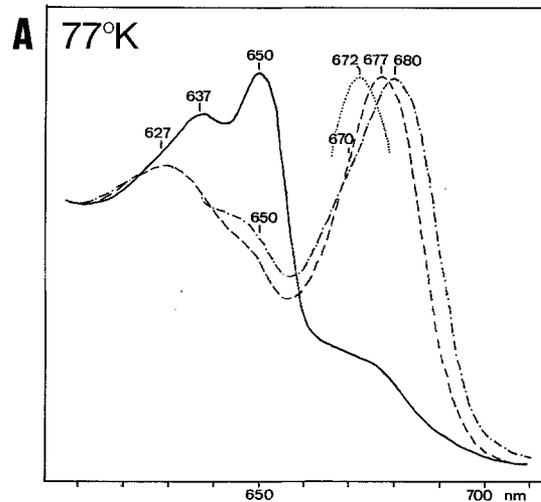
The 77°K fluorescence spectra of etiolated radish cotyledons exhibited peaks at 657 and 630 nm (Fig. 1). The relative height of these peaks changed during growth in the dark. At the beginning, from day 1 to day 6 following germination, the intensity of the fluorescence emission at 657 nm ( $F_{657}$ ) decreased compared to that at 630 nm ( $F_{630}$ ). Later, between day 6 and day 10,  $F_{657}$  increased, whereas after day 10, no further change of the spectrum occurred (Fig. 3a, control).

Cotyledons from seedlings grown in the presence of added kinetin showed smaller changes of the relative heights of the fluorescence peaks. Up to day 8 after germination the ratio  $F_{657}/F_{630}$  was higher in kinetin-treated plants, but afterward this ratio remained at a lower level (Fig. 3a, kinetin).

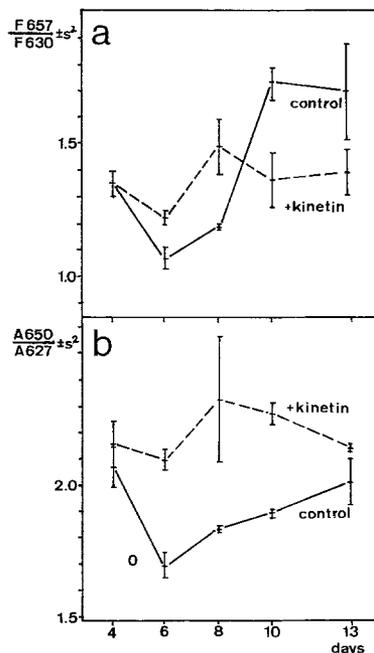
Absorption spectra of etiolated cotyledons had a main peak at 650 nm and a side peak at 637 nm (Fig. 2). In the controls the absorption at 650 nm



**Fig. 1.** Low-temperature (77°K) fluorescence spectra of etiolated *Raphanus* cotyledons before (—), immediately (---), 30 s (-·-·-), and 30 min (····) after one 1-ms flash. The 30-min curve is only indicative of the position of the long wavelength peak



**Fig. 2.** Low-temperature (77°K) absorption spectra of etiolated *Raphanus* cotyledons before (—), immediately (---), 30 s (-·-·-), and 30 min (····) after one 1-ms flash. The 30-min curve is only indicative of the position of the long wavelength peak



**Fig. 3a and b.** Relative amount of the phototransformable form of protochlorophyll(ide) in etiolated *Raphanus* cotyledons for different ages of seedlings grown in the absence (control) or presence of kinetin. **a** Ratio of the fluorescence (77°K) at 657 nm to that at 630 nm ( $F_{657}/F_{630}$ ). **b** Ratio of the absorption (77°K) at 650 nm to that at 627 nm ( $A_{650}/A_{627}$ )

( $A_{650}$ ) decreased relative to that at 627 nm ( $A_{627}$ ) from day 4 to day 6 following germination; afterward the  $A_{650}/A_{627}$  ratio rose slowly to day 13 (Fig. 3b, control).

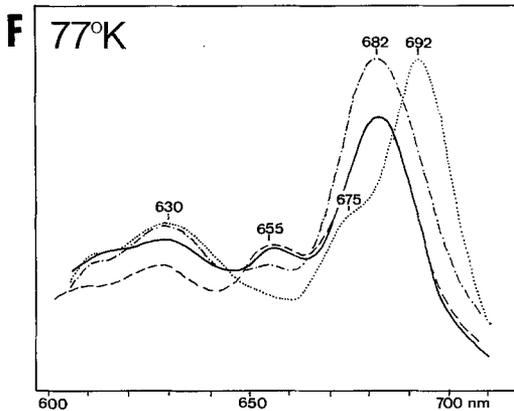


Fig. 4. Low-temperature (77°K) fluorescence spectra of etiolated control *Raphanus* cotyledons 30 min after one 1-ms flash at different seedling ages (4 days ----, 6 days —, 8 days - - - - , 13 days ·····)

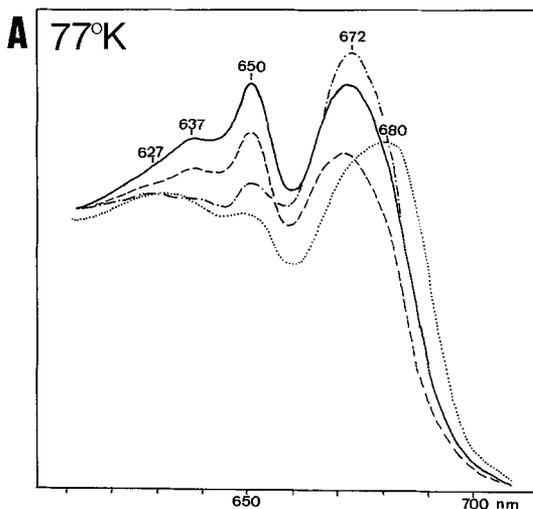


Fig. 5. Low-temperature (77°K) absorption spectra of etiolated control *Raphanus* cotyledons 30 min after one 1-ms flash at different seedling ages (4 days ----, 6 days —, 8 days - - - - , 13 days ·····)

Cotyledons from seedlings grown in the presence of kinetin showed higher  $A_{650}$  to  $A_{627}$  ratios than the controls. This was especially pronounced from day 6 to day 10 following germination (Fig. 3b, kinetin).

## 2. Effect of One Flash

### 2.1. Spectra Registered Immediately, or 30 s after a First 1-ms Flash

Immediately after a first 1-ms flash (see Methods) the 77°K fluorescence emission maxima were found at 630 and 688 nm (Fig. 1). The peaks of the absorp-

tion spectra were correspondingly found at 627 and 677 nm; sometimes a shoulder remained at 650 nm (Fig. 2).

Cotyledons frozen 30 s after the first flash showed fluorescence emission maxima at 630 and 692 nm with a shoulder at 675 nm (Fig. 1). The corresponding absorption peaks were found at 627 and 680 nm with shoulders at 650 and 670 nm (Fig. 2).

All 77°K fluorescence and absorption spectra of cotyledons belonging to kinetin-treated seedlings or to control seedlings showed the same peaks, after one individual flash and 30 s later.

The characteristics of the spectra did not depend on the age of the seedlings.

### 2.2. Spectra Registered 30 min after a First 2-ms Flash

After a 30-min dark period the spectra were changed again, and the position change of the peaks became age dependent.

#### 2.2.1. Young Cotyledons (4 or 6-day-old Seedlings).

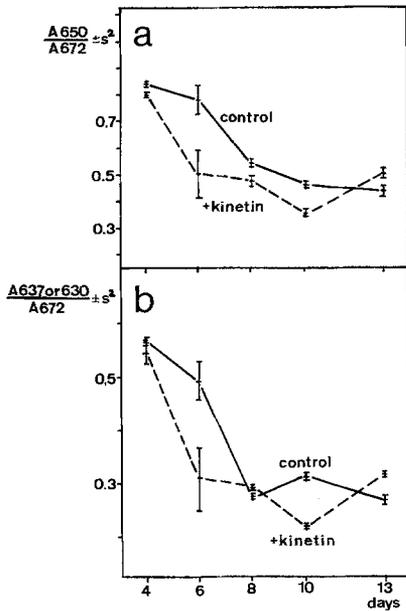
In young cotyledons the fluorescence emission maxima (77°K) seen 30 min after a first 1-ms flash (see Methods) were found at 655 nm (Fig. 4, 4 and 6 days). The emission at 682 nm was always more intense than that at 630 nm (1.3 to 2.5 times higher). Thirty min after the first 1-ms flash the absorption spectra of young cotyledons showed main peaks at 672 and 650 nm, as well as an additional side peak at 637 nm (Fig. 5, 4 and 6 days).

Comparing control and kinetin-treated young cotyledons showed that 30 min after the first flash there was less absorption at 650 ( $A_{650}$ ) and 637 nm ( $A_{637}$ ) in 6-day-old cotyledons grown in the presence of kinetin (Fig. 6).

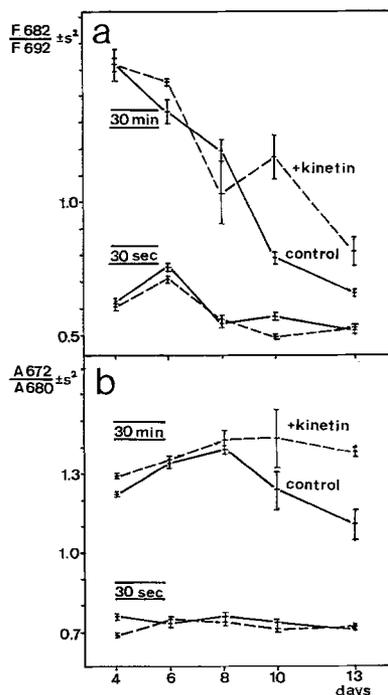
#### 2.2.2. Older Cotyledons (8, 10, or 13-day-old Seedlings).

With increasing cotyledon age, the absorbance at 650 and 637 nm did reappear more and more slowly within the 30-min dark period following a flash (Fig. 5, 8 and 13 days). This was true both for control and kinetin-treated plants.

The slow shift of the main peaks of the absorption and fluorescence spectra from  $A_{680}$  to  $A_{672}$  and from  $F_{692}$  to  $F_{682}$ , which occurs in darkness after an illumination (Shibata shift: Shibata, 1957), was accomplished earlier in kinetin-treated than in control cotyledons at day 10 and day 13 following germination. Cotyledons from 10 or 13-day-old control seedlings needed up to 2 h to accomplish the shift. In the 13-day controls, after a dark phase of 30 min, the peaks remained in the position they were in 30 sec after the flash ( $F_{692}$ ,  $A_{680}$ ), or their positions were



**Fig. 6a and b.** Relative amount of the protochlorophyll(ide) resynthesized in etiolated *Raphanus* cotyledons after one 1-ms flash during a dark period of 30 min at different ages of seedlings grown in the absence (control) or presence of kinetin. **a** Ratio of the absorption (77°K) at 650 nm to that at 672 nm. **b** Ratio of the absorption (77°K) at 637 nm (younger cotyledons) or 630 nm (older cotyledons) to that at 672 nm



**Fig. 7a and b.** Relative accomplishment of the Shibata shift in *Raphanus* cotyledons at different ages of the seedlings grown in the absence (control) or presence of kinetin. The accomplishment of the shift is expressed by the ratios of the 77°K fluorescence **a** and absorption **b** maxima expected after ( $F_{682}$ ,  $A_{672}$ ) and before ( $F_{692}$ ,  $A_{680}$ ) the shift. The ratios for cotyledons frozen 30 s after a single 1-ms flash (before the Shibata shift) are compared to the ratios for cotyledons frozen 30 min later

intermediary between  $F_{692}$ - $F_{682}$  and  $A_{680}$ - $A_{672}$  (Figs. 4 and 5, 13 days).

The ability to perform the Shibata shift was expressed by the ratio of the peak heights at the position where the absorption and fluorescence maxima were to be expected before ( $F_{692}$ ,  $A_{680}$ ) and after ( $F_{682}$ ,  $A_{672}$ ) the accomplishment of the shift. The values of the  $F_{682}/F_{692}$  and  $A_{672}/A_{680}$  ratios before the shift were estimated after 30 s darkness (Shibata shift = 0%). After 30 min darkness the  $F_{682}/F_{692}$  ratio decreased with increasing seedling age in control and in kinetin-treated plants, showing that the amount of the shifted form decreased relative to that of the unshifted form (Fig. 7a). There was no difference in the  $F_{682}/F_{692}$  ratios between kinetin-treated and control plants up to day 8. However, for seedlings older than 8 days the  $F_{682}/F_{692}$  ratios were higher in plants grown in the presence of kinetin, indicating that the Shibata shift was preserved by kinetin.

Absorption spectra also demonstrated the preservation of the Shibata shift by kinetin in older cotyledons. The  $A_{672}/A_{680}$  ratios found 30 min after a flash decreased considerably in control seedlings older than 8 days. However, in kinetin-treated plants, there was a minimal change in the ratio (Fig. 7b).

## Discussion

The 77°K fluorescence and absorption spectra described here for etiolated *Raphanus* cotyledons were described previously in *Phaseolus* (Goedheer, 1967; Sironval et al., 1968). The fluorescence at 657 nm ( $F_{657}$ ) is due to two phototransformable protochlorophyll(ide) forms absorbing at 650 ( $A_{650}$ ) and 637 nm ( $A_{637}$ ) respectively ( $P_{657-650}$  and  $P_{645-637}$ , Boardman, 1966; Boardman et al., 1970; Kahn et al., 1970; Bovey et al., 1974), whereas to explain the fluorescence at 630 nm ( $F_{630}$ ), a hidden maximum at 627 nm ( $A_{627}$ ) belonging to a nonphototransformable protochlorophyll(ide)  $P_{627-630}$  (Boardman et al., 1970; Kahn et al., 1970; Bovey et al., 1974) must be postulated.

The rise in the amount of the long wavelength protochlorophyll(ide) form  $P_{657-650}$  during growth, which appears to start in *Raphanus* 6 days after germination (Fig. 3a, b) was also described for *Phaseolus* seedlings (Boardman et al., 1970; Thorne, 1971; Klein and Schiff, 1972). During the accumulation of  $P_{657-650}$  the formation and enlargement of the prolamellar body was observed (Butler and Briggs, 1966; Klein and Schiff, 1972; Broderson, 1976). In agreement with other findings (Boardman et al., 1970; Thorne, 1971), the increase of the  $F_{657}/F_{630}$  ratio was maintained in *Raphanus* only up to a certain age. However, the

slight decrease of the relative amount of the long wavelength form  $P_{657-650}$  which we have found in *Raphanus* between day 4 and day 6 after germination, has never been described elsewhere.

The higher  $A_{650}/A_{627}$  ratios in kinetin-treated seedlings, as compared to control seedlings, especially between day 6 and day 10, indicate that kinetin enhances the accumulation of phototransformable  $P_{657-650}$ . It is also remarkable that at day 10 and day 13 the ratio  $A_{650}/A_{627}$  was found to be smaller while that of  $F_{657}/F_{630}$  was higher in the controls than in kinetin-treated plants (Fig. 3a, b). This may be understood in admitting a more efficient energy transfer from  $P_{645-637}$  to  $P_{657-650}$  (Boardman et al., 1970; Kahn et al., 1970; Bovey et al., 1974) in control cotyledons when the seedlings are older than 8 days.

The 77°K fluorescence and absorption spectra registered immediately after one flash show peaks characterizing the chlorophyllide-protein complex  $P_{688-676}$  (Sironval and Michel, 1967; Sironval et al., 1968; Sironval and Kuiper, 1972) and the nonphototransformable  $P_{630-627}$  (Boardman et al., 1970; Kahn et al., 1970; Bovey et al., 1974). During 30 s of darkness the long wavelength peak undergoes the rapid shift ( $F_{688}/A_{677} \rightarrow F_{692}/A_{680}$ , Sironval and Michel, 1967; Sironval and Kuiper, 1972). When the dark period is lengthened to 30 min the protochlorophyll(ide) absorption peaks reappear at 650 and 637 nm, especially in young seedlings (Fig. 5, 4 and 6 days). The fluorescence emission at 655 nm reappears to a lesser extent as a result of the energy transfer from  $P_{657-650}$  and  $P_{645-637}$  to  $P_{688-676}$  (Butler, 1961; Kahn et al., 1970; Thorne, 1971; Fig. 4, 4 and 6 days). The ability to resynthesize protochlorophyll(ide) in the dark after illumination diminished with increasing age, as already pointed out in the literature (Shibata, 1957; Butler, 1961; Akoyunoglou and Siegelman, 1968; Fig. 5).

This is true both for control and kinetin-treated *Raphanus* seedlings. At day 6 after germination, however, seedlings grown in the presence of kinetin resynthesized in the dark smaller amounts of protochlorophyll(ide) than controls after a 1-ms flash of light (Fig. 6a, b). This may be related to a particular concentration of endogenous cytokinin in the cotyledons at day 6.

Shlyk (Shlyk and Averina, 1969; Shlyk et al., 1970) found that excised barley leaves illuminated for 24 h in the presence of kinetin were enriched in protochlorophyll(ide). At variance with our experiments, Shlyk used a long 24-h illumination time. According to Fletcher (Fletcher and McCullagh, 1971a; Fletcher et al., 1973) cytokinins increase protochlorophyll(ide) biosynthesis only when  $\delta$ -aminolevulinic acid synthetase is also stimulated by light. The single

1-ms flash used here apparently does not change the activity of the enzyme.

The ability to perform the Shibata shift (Shibata, 1957) decreased with seedling age in *Raphanus* control seedlings (Fig. 7a, b) as already shown by Akoyunoglou (Akoyunoglou and Siegelman, 1968; Akoyunoglou and Michalopoulos, 1971) in *Phaseolus*. In *Raphanus* the amount of the shifted fluorescence form at 682 nm decreased continuously relative to the nonshifted fluorescence form at 692 nm with increasing age (Fig. 7a), whereas the shifted 672 nm absorption decreases relative to the nonshifted 680 nm absorption after day 8 only. An explanation for this might be provided by taking into account changes in energy transfer efficiency from one pigment-protein complex to the other.

In 10- and 13-day-old cotyledons, the Shibata shift is more rapid when the seedlings are grown in the presence of kinetin (Fig. 7a, b, kinetin). This preservation of the Shibata shift may be considered a further indication for the general fact that aging processes proceed more slowly (Richmond and Lang, 1957; Mothes, 1961) in kinetin-treated cells.

This work was sponsored by a NATO fellowship given to C. Buschmann.

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Received 14 August; accepted 24 October 1977