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Chlorophylls and carotenoids in a fully habituated nonorganogenic callus of *Beta vulgaris*

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Abstract

A fully habituated (H) nonorganogenic sugar beet callus, subcultured in the light, did not contain detectable chlorophyll (Chl) nor carotenoid (Car). It accumulated some Car in the dark. Fluorescence spectra indicated that this H callus also accumulated some protochlorophyllide which, however, was not well integrated into the protochlorophyllide-NADPH-photoreductase complex, and therefore not transformed into chlorophyllide in the light. The H callus showed no variable fluorescence which indicated absence of photosynthesis, and therefore it suggested a full heterotrophic behaviour of this peculiar callus line. A green hormone-dependent callus of the same sugar beet had normal fluorescence spectra and kinetics comparable to those of a green leaf.

Introduction

A whitish fully habituated nonorganogenic callus of sugar beet has been described with many characteristics of a vitrified tissue: a lower content of Chl than in a normal (auxin and cytokinin dependent) callus of the same sugar beet strain, hyperhydricity, and hypolignification (Crevecoeur *et al.* 1987, Gaspar *et al.* 1988).

Cells of this callus furthermore exhibit many features of cancerous cells (Gaspar *et al.* 1991): monoclonal origin (Kevers *et al.* 1981), abnormally shaped large polyploid nuclei with several nucleoli (Hagège *et al.* 1992a), disturbed sugar metabolism (Bisbis *et al.* 1993) and deviated nitrogen metabolism leading to polyamine accumulation (Hagège *et al.* 1990, Le Dily *et al.* 1993a), elevated levels of diacylglycerol and inositol phosphates (Feutry *et al.*, unpublished). These poorly differentiated cells also exhibit incompletely structured plastids and mitochondria (Crevecoeur *et al.* 1992) with a general deficiency of the tetrapyrrole-compounds,

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peroxidase, catalase, and cytochromes besides Chl (Hagège *et al.* 1992b, Le Dily *et al.* 1993b). Investigating the energetical economy of such, nevertheless actively dividing cancer cells, the present work aims at precising their photosynthetic capacity. The Chl and Car levels will be reestimated in calli cultured in the light and in the dark taking a normal green callus and/or a leaf of the mother plant as controls but the kinetics of Chl fluorescence will be mainly exploited because their changes well correspond to net photosynthetic rates (Capellades *et al.* 1990).

Material and methods

Plant material and cultures: Experimental conditions for obtaining normal (N) and habituated (H) callus of sugar beet (*Beta vulgaris* L. var. *altissima*), and for maintaining these tissues in solid stock cultures under light (16 h photoperiod of *Sylvania Grolux* fluorescent light providing 17 W m^{-2}, 25 °C) have been reported elsewhere (Kevers *et al.* 1981). Such calli when subcultured (transfer of seven 200 to 500 mg callus pieces, in 10-cm plastic Petri dishes) every 3 weeks on their respective solid medium [basal medium without plant growth regulators for habituated lines, but supplemented with 0.1 g m^{-3} 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.1 g m^{-3} benzyladenine (BAP) for normal lines] have remained apparently unchanged for at least 12 years.

Chlorophylls and carotenoids extraction and estimation: 1 g of 14 d-old callus was ground in a cold mortar in 80 % v/v acetone containing sodium bicarbonate (for pH stability). The resultant suspension was centrifuged for 15 to 20 min at 10 000 *g*. Chl and Car were spectrophotometrically determined in the supernatant according to the equations of Arnon (1949) and Liaaen-Jensen and Jensen (1971). The absorption spectra of the acetonic extracts were registered from 400 to 700 nm using the spectrophotometer *Varian*, model *Cary 17*.

Fluorescence spectra of intact cells were registered at 77 K using a spectrofluorimeter according to Sironval *et al.* (1968) with excitation wavelength of 436 nm (half-band width = 10 mm).

Variable fluorescence kinetics were measured at room temperature on small pieces of calli kept in the dark for 15 to 30 min. The excitation radiation at 632.8 nm was provided by the He-Ne laser (50 W m^{-2}). A multi-branched fiber optic guide was used to transmit the exciting radiation from the laser to the sample and the fluorescence from the sample to the detector (photomultiplier *EM 19558B*). Fluorescence emission was measured at 691 nm. The intensity of the ground fluorescence (F_0) was measured using short laser pulses obtained by means of a rotating disk system comprising a slit in front of the laser. In these conditions F_0 was measured after 0.1 ms.

Results

Absorption spectra and chlorophyll and carotenoid contents: The absorption spectrum of the acetonic extract of N callus was similar to that obtained from a green sugar beet leaf, with a main red band at 663 nm (Fig. 1). Both the Chl and Car contents of the N callus decreased when transferred to darkness (Table 1). The H callus produced a turbid extract only with traces of Chl and Car pigments (Fig. 1, Table 1) under normal irradiance. It was repeatedly found that H cells still contained traces of Car when transferred in darkness (Table 1).

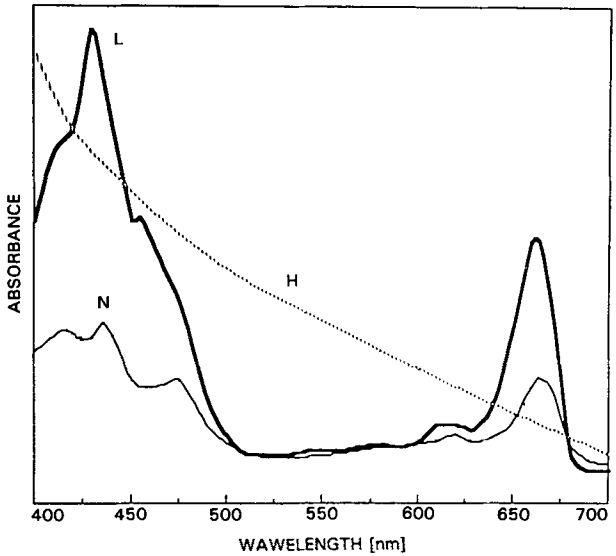

Fig. 1. Absorption spectra of acetone extracts of sugar beet N callus (*N*), green leaf (*L*) and H callus (*H*).

Table 1. Chlorophyll (*a*+*b*) and total carotenoid contents in H and N calli of sugar beet (means ± SE; $n = 6$).

		H callus	N callus
11 d darkness	Chl (*a*+*b*) [mg kg^{-1}(f.m.)]	0	7.4 ± 0.9
	Car [mg kg^{-1}(f.m.)]	0.07 ± 0.01	0.4 ± 0.04
11 d light	Chl (*a*+*b*) [mg kg^{-1}(f.m.)]	0	12.9 ± 0.9
	Car [mg kg^{-1}(f.m.)]	0	2.0 ± 0.6

Fluorescence spectra: The fluorescence emission spectrum of the N callus grown as usual in the light presented three bands at 732, 695 and 686 nm, while after 11 d in the dark an increase of the band at 732 nm and a decrease of the two bands at 686 and 695 nm were observed (Fig. 2*N*). The spectrum of the H callus grown in the light

showed a low intensity emission at 683 nm (Fig. 2*H*). After transfer to darkness for 11 d, the H callus cells still showed the band at 683 nm but it was relatively lower and less intense than a specific band at 635 nm appearing under darkness. This band at 635 nm decreased after exposure (10 or 90 nm) of the H cells to continuous "white light", but without a concomitant increase of the 683 nm band.

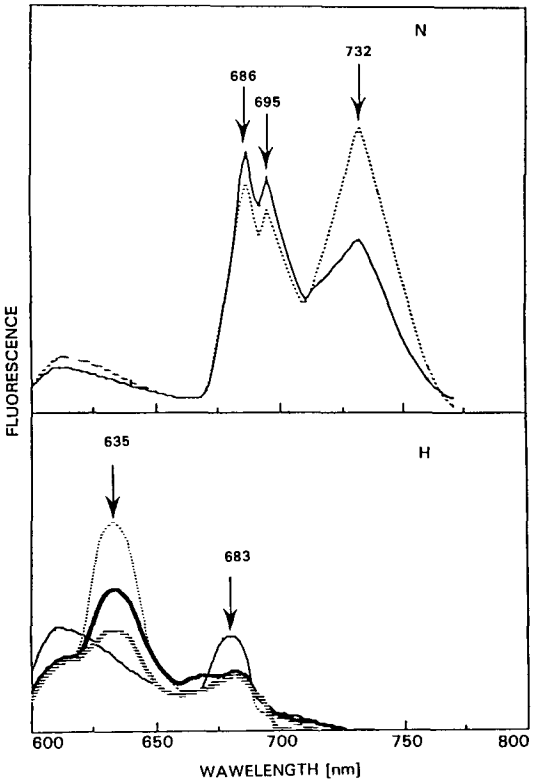

Fig. 2. Fluorescence spectra of N and H calli after 11 d in the light (*full line*) or in the dark (*dotted line*) or after 11 d in the dark followed by 10 min (*fat line*) or by 90 min (*dashed line*) of continuous irradiation.

Variable fluorescence: Typical variation of the room temperature fluorescence intensity were observed with the N callus, with $F_V = 0.6 \pm 0.2$. The H callus containing no Chl, showed only constant fluorescence intensity (Fig. 3).

Discussion

The Chl content of the N callus [12.9 mg kg^{-1}(f.m.)], measured in 1993 was the same as what measured in 1987 (Crevecoeur *et al.* 1987). It has fallen from 3.8 mg kg^{-1} to zero with the successive subcultures of the H callus in the same period of time. The H callus grown under light did not contain Car but some Car were accumulated when

cultured under darkness. A protective effect of Car against photooxidation and the generated free radicals (Krinsky and Deneke 1982) in the light thus cannot be expected in the H callus.

The N callus showed a typical fluorescence spectrum of a green leaf with three characteristic emission bands at 686, 695 and 732 nm indicating that it has an organized and functional photosynthetic apparatus. The H callus showed a relatively weaker emission band at 683 nm. Then, in order to examine if this callus would produce the Chl precursor in darkness, we investigated the changes in fluorescence spectra after 11 d in the dark. We could observe an accumulation of a pigment emitting at 635 nm (Fig. 2*H*). After 10 or 90 min continuous "white light" the intensity of the band decreased. This pigment emitting at 635 nm could be a protochlorophyllide not well integrated into a photoactive protochlorophyllide-NADH-photoreductase complex, and therefore not transformed to Chl in the light

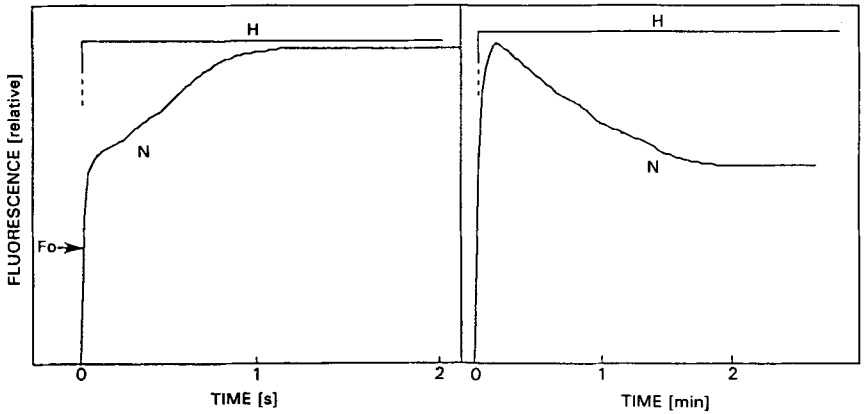

Fig. 3. Variable fluorescence kinetics of N and H calli in the s and min time scale.

(Dujardin and Sironval 1970). In etiolated plants indeed, the photochlorophyllide-NADPH-photoreductase complex has a characteristic band at 657 nm, that is replaced by a chlorophyllide emission band at 688 nm after a short illumination. The 635 nm emission is probably due to an accumulation of the protochlorophyllide which is not transformed into Chl within a short time of illumination. Chl fluorescence kinetics is associated with the primary photochemical events (Sivac and Walker 1986). In general, the study of variable fluorescence provides important informations on the functional state of the photosynthetic apparatus and more specifically on the efficiency of photosystem 2 (Krause and Weis 1991). The absence of detectable variable fluorescence in the H callus indicated that no photosynthetic electron transport occurred in this callus. On the contrary, the N callus showed normal fluorescence variations associated with photosynthesis. This is well related to the plastid structure of the H cells which is altered, containing starch grains and few grana (Crevecoeur *et al.* 1992). The overall data thus actually conclude to a full heterotrophic behaviour of the H callus. In order to further explore the energetics of this peculiar cell line, investigations are performed on the capability to incorporate $^{14}CO_2$ and on the metabolization of ^{14}C-sucrose. The relationships between the

photosynthetic properties of the H callus and other biochemical characteristics (see Introduction) are also investigated.

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