

Effect of abscisic acid on differentiation and ribulose diphosphate carboxylase of chloroplasts

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Daucus carota tissues were grown on Murashige-Skoog medium (MS) at different concentrations with abscisic acid (ABA). Seven bands of chloroplast fractions were obtained on a sucrose gradient. At 10^{-5} M, ABA highly increased chlorophyll and protein nitrogen content of medium density chloroplasts. With increasing age of the tissues, the most active chloroplasts according to their $^{14}\text{CO}_2$ fixation were found in smaller numbers. When treated with 10^{-5} M ABA, 34 day-old tissues cultivated in vitro showed the chloroplast pattern of 110 day-old tissues. The effect of ABA—given to the tissues during a short pretreatment or continuously present in the culture medium—on the ribulose diphosphate carboxylase activity was analysed. It was found that ABA at 10^{-5} M strongly inhibited $^{14}\text{CO}_2$ fixation.

Key words: ABA — Chloroplasts — Differentiation enzyme — RuDP-carboxylase — Tissue culture.

Analyses of the ABA effects on several tissues cultivated in vitro have given rise to a number of publications (12). In our Institute the changes in the ultrastructure—induced by ABA—have been most particularly studied in the tissues of *Rubus hispidus* cultivated in vitro (11, 13) especially in relation to their growth. Some changes in plastid structures when these tissues were exposed to light for several days have been briefly reported. That is why experiments, using carrot tissues (rich in chloroplasts), were conducted to test the direct action of ABA on the chloroplasts and their chlorophyll content.

Materials and methods

Materials

Daucus carota tissue cultures (4, 8-10) were maintained on Murashige-Skoog (MS) medium containing 1.7×10^{-6} M IAA and 6×10^{-9} M kinetin. Experiments using different concentrations of abscisic acid (ABA) were conducted with 34 day-old cultures of tissues cultivated in vitro. The activity of ribulose diphosphate (RuDP) carboxylase was analysed using isolated chloroplasts from 48 day-old cultures with or without ABA (10^{-5} M). Similar analyses were done on isolated chloroplasts from 110 day-old cultures pretreated with or without ABA (10^{-5} M).

Methods

Each step of the extraction and isolation of chloroplasts until the osmotic shock

was checked, on an untreated sample, by electron microscopy (Dr. R. M. Hofer, in a further paper). After this check showed that the technique used yielded mainly structurally intact chloroplasts, as routine electron microscopy examination of every chloroplast preparation would be extremely time-consuming, we checked the pellet placed on a discontinuous sucrose gradient under light microscope and phase contrast. Chloroplasts in this pellet were of several sizes, smaller than those of higher plants, and predominantly of either class I or class II as described by Spencer and Unt (16), and Spencer and Wildman (15). Some of them appeared opaque, the grana difficult to differentiate, the margin of the chloroplasts well defined as described by Leech (6) and Bourdu et al. (2) for membrane-bound chloroplasts; others appeared with a very granular appearance, diffused boundaries, presumably chloroplasts in which the external membrane was not complete, not refractive.

Extraction: Tissues were ground with a mortar and pestle in a semi-frozen extraction buffer (0.02 M glycyl-glycine + 5 mM $\text{MgCl}_2 \cdot 6 \text{H}_2\text{O}$ + 1 mM mercapto-ethanol + 0.33 M saccharose) (5), and filtered through 4 layers of muslin and 2 layers of nylon gauze (pore size: 50 μm) to remove cellular debris. The muslin was pressed and the filtrate collected and centrifuged (12,500 $\times g$, 2 min). The pellet, corresponding to nearly all the chloroplasts, after light microscope examination, was placed on a discontinuous sucrose gradient (33–42–51–57–60–63–66–70% w/w) and centrifuged (23,000 $\times g$, 90 min, 5°C) using the ultracentrifuge Christ Omega 2.

Isolation of chloroplasts: Different types of chloroplasts were separated according to their density. The fractions were successively collected and their index of refraction at 25°C measured by refractometry. These fractions were diluted three times with the extraction buffer, without sucrose, and centrifuged (2 min, 7000 $\times g$). The pellets—containing isolated chloroplasts—were taken up in the extraction buffer without sucrose in order to burst (osmotically) the chloroplasts, as Ru-DP carboxylase is found inside the chloroplast (14, 17), and to allow substrates to react with active enzyme sites (2).

Ru-DP carboxylase activity: 0.3 ml of the above fractions were mixed with 10 μCi $\text{NaH}^{14}\text{CO}_3$ (50 μl), 0.5 μmole Ru-DP (disodium salt), 0.4 ml incubation buffer (0.25 mM glycyl-glycine, 2.5 mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 75 mM NaHCO_3 , mercapto-ethanol 5 $\mu\text{l}/250$ ml; pH 7.8) (5) in scintillation vials, which were then incubated (1 hr, 25°C) in white light (Philips: TL 40 w/33; blanc super; 1.5×10^8 lux).

The reaction was stopped by adding 0.2 ml of 6 N acetic acid. The vials were then flushed for 4 hr with air to remove the mineral-labelled CO_2 in excess, and the samples dried. Then 2.5 ml of H_2O and 10 ml of Bray's scintillation fluid were added to the dry residues and the radioactivity of each sample was measured in a liquid scintillation counter.

Analytical methods: Chlorophyll content was estimated according to a method previously described (1, 3). An aliquot of the aqueous extract was diluted with acetone to adjust the water content to 20% (v/v). The absorbance was measured with a recording spectrophotometer at 663, 645 and 652 nm.

Protein nitrogen content was estimated according to the Kjeldahl method. To 1 ml of each fraction, 5 ml of 10% TCA were added. Proteins were precipitated at 4°C for 18 hr and the tubes were then centrifuged (4,200 $\times g$, 30 min). The supernatant containing the non-protein nitrogen was discarded. To the pellets were added respectively 1 ml concentrated H_2SO_4 , a small amount of selenium containing

catalyst and a few glass beads. The pellets were then hydrolyzed for 18 hr at 330°C in a thermobloc (Lievisch, Planta Jenny). Ammonia was distilled according to the Parnas-Wagner method and collected in 0.05 N H₂SO₄. The protein nitrogen was determined by a back titration of the H₂SO₄ with 0.02 N NaOH.

Results and discussion

First, experiments were performed in order to study changes in the chloroplast distribution with the increasing age of the tissues.

Seven bands were observed in the sucrose gradient (Fig. 1A); they can be classified into three groups, according to their density ρ :

- Group I : light chloroplasts, density 1.141–1.213 (zones 33 to 42% sucrose)
- Group II : medium chloroplasts, density 1.257–1.280 (zones 51 to 57% sucrose)
- Group III : heavy chloroplasts, density 1.304–1.322 (zones 60 and 63 to 66% sucrose).

In young tissues (27 days), there were more light chloroplasts (group I). Those of group II were almost nonexistent, while those of group III were of few importance.

When the tissues became older (48 days), the number of chloroplasts in groups I and III increased. When the tissues were old (110 days), chloroplasts from groups I and III were at about the same level as in the 48 day-old tissues; in contrast, chloroplasts of group II became very abundant. There were a great many middle and high density chloroplasts.

The distribution patterns of chloroplasts, obtained from tissues treated with ABA, were changed in the following way (Fig. 1B):

—with 10⁻⁶ M ABA, the chlorophyll (Fig. 2) and protein (Fig. 3) content of group I

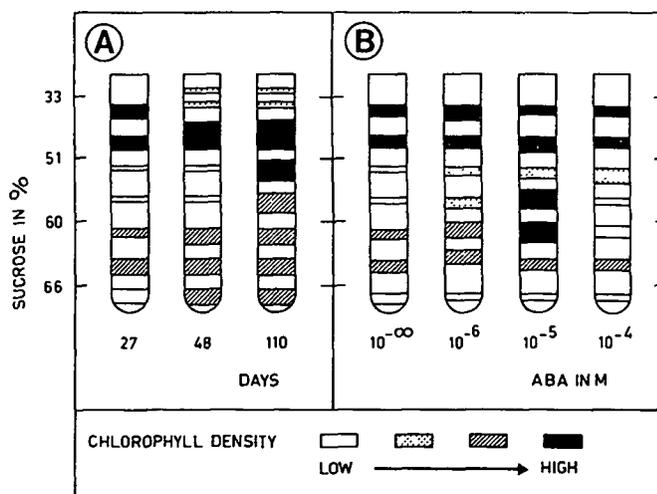


Fig. 1. Changes in chloroplast distribution patterns (separation in sucrose gradient). (A) With increasing age of the tissues (in days) cultivated in vitro. (B) Effect of ABA (at several concentrations). Age of cultures: 34 days.

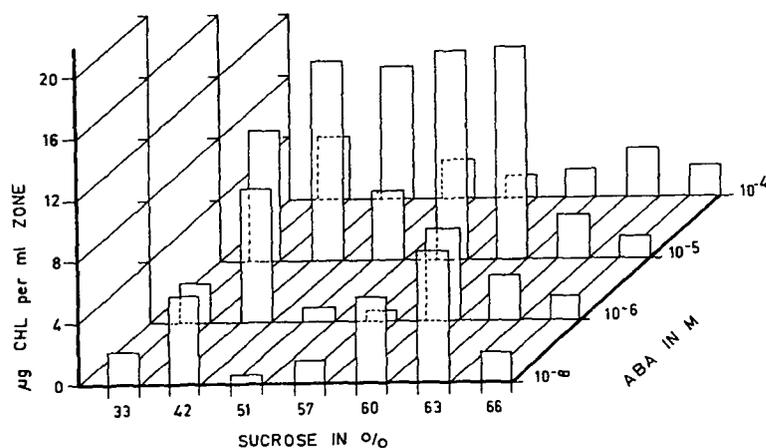


Fig. 2. Chlorophyll content (in $\mu\text{g ml zone}$) of the seven isolated chloroplast sucrose zones. Chloroplasts were prepared from carrot tissues cultivated on Murashige and Skoog medium in the presence of ABA at several concentrations. Age of cultures: 34 days.

chloroplasts increased and became higher than that of group III. Group II chloroplasts were still very few.

— 10^{-5} M ABA, the chlorophyll and protein levels of group I and group II chloroplasts was clearly enhanced. As can be seen in Fig. 2, chlorophyll concentration (in $\mu\text{g/ml}$) rose from 5.81 to 13.00 in the 42% zone. In the 57% zone (a very thin band almost undistinguishable in the control), the chlorophyll content rose from 1.44 to 13.49 $\mu\text{g/ml}$. The 51% zone was stimulated as well as the 60%

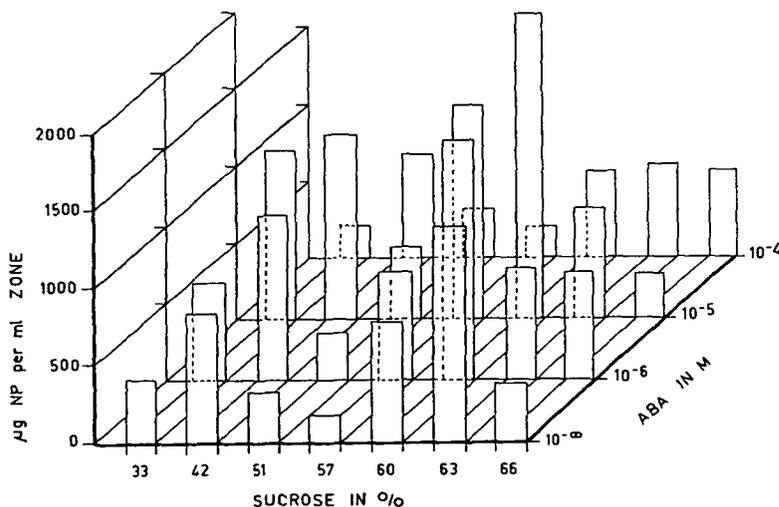


Fig. 3. Protein content (in $\mu\text{g ml zone}$) of the seven isolated chloroplast sucrose zones. Chloroplasts were prepared from carrot tissues cultivated on Murashige and Skoog medium in the presence of ABA at several concentrations. See Fig. 2.

one, in which chlorophyll content was increased from 5.78 $\mu\text{g/ml}$ to 13.78 $\mu\text{g/ml}$. In contrast the 63% and 66% zones "degenerated". The protein content (Fig. 3) was found to change in a similar way.

—with 10^{-4} M ABA, stimulation of the light chloroplasts of group I was reduced, compared to the effect of 10^{-5} M ABA while group III zones—60, 63 and 66% sucrose—were destroyed. The chlorophyll (Fig. 2) and protein (Fig. 3) content for these zones was found to be decreased. Such results were expected, since tissues showed necrosis in the presence of ABA at a concentration of 10^{-4} M.

Next, changes in the chloroplast Ru-DP carboxylases were tested (Tables 1 and 2). As more significant results were obtained with 10^{-5} M ABA, a large number of tissue explants were cultivated (fresh weight=300 g) in Erlenmeyer flasks normal MS medium with or without ABA at 10^{-5} M. The Results are reported in Table 1, as nanomoles of CO_2 incorporated per mg of Chl, per ml and per hr, versus sucrose density. As can be seen, ABA has an inhibitory effect on all the zones except on the high density group of chloroplasts.

Such an inhibition of Ru-DP carboxylase activity by ABA was also obtained for the 110 day-old tissues, pretreated 17 hr in a phosphate buffer pH 6.1 (Table 2) containing 10^{-5} M ABA.

When comparing Ru-DP carboxylase activity of untreated 48 day-old and 110 day-old tissues, it may be noticed that CO_2 fixation characterised mainly the chloroplasts of 33, 42, 51, 57% zones. As a matter of fact, in old tissues, the larger number of heavy chloroplasts (57, 60, 63 and 66% sucrose), less active in CO_2 fixation, leads the tissues towards necrosis.

Table 1 CO_2 incorporated in organic compounds expressed in nm for two parameters (per mg of CHL and per mg of NP) in zones of isolated chloroplasts from carrot tissues (cultivated 48 days on the Murashige and Skoog medium, with or without $1 \cdot 10^{-5}$ M ABA)

		Concentration of sucrose in %						
		33	42	51	57	60	63	66
Average index of refraction ^a		1.3865	1.4139	1.4310	1.4390	1.4471	1.4526	1.4559
	±	0.0030	0.0047	0.0028	0.0014	0.0041	0.0036	0.0017
Density D_4^{20} (ρ)		1.141	1.213	1.257	1.280	1.304	1.316	1.322
No ABA	a	35.5	92.0	4.6	2.8	28.6	14.2	16.5
	b	1890	4060	507	599	1710	1290	1290
	CO_2 (1)	716	815	821	626	243	301	257
	CO_2 (2)	13.5	18.5	7.5	2.9	4.0	3.3	3.3
+ABA	a	127.0	43.5	8.2	24.0	28.4	11.0	8.3
	b	4380	1240	622	1290	1840	1590	1240
	CO_2 (1)	475	268	311	281	243	449	356
	CO_2 (2)	13.8	9.3	4.1	5.2	3.7	5.3	2.4

a: μg of CHL/ml b: μg of NP/ml

CO_2 (1): CO_2 per mg of CHL/ml/hr CO_2 (2): CO_2 per mg of NP/ml/hr

^a Evaluation for 9 experiments

Relative standard error in the determination of chlorophylls and protein nitrogen respectively: 6% and 0.25%.

Table 2 CO_2 incorporated in organic compounds expressed in nm for two parameters (per mg of CHL and per mg of NP) in zones of isolated chloroplasts from carrot tissues (cultivated 110 days on the Murashige and Skoog medium) and pretreated in a phosphate buffer, pH 6.1, with or without $1 \cdot 10^{-5}$ M ABA for 17 hr

		Concentration of sucrose in %						
		33	42	51	57	60	63	66
No ABA	a	79.0	59.0	44.5	38.0	24.4	55.2	42.8
	b	2000	1070	2120	1520	783	1570	1610
	CO ₂ ⁽¹⁾	1070	344	2480	885.0	237.2	149.0	155.6
	CO ₂ ⁽²⁾	42.0	19.0	52.2	22.1	7.4	5.2	4.1
+ABA	a	103.0	98.6	91.2	24.9	18.4	15.9	14.0
	b	1750	830	2030	740	740	1110	900
	CO ₂ ⁽¹⁾	602	282	205	332	377	465	415
	CO ₂ ⁽²⁾	35.4	33.5	9.2	11.2	9.5	6.7	6.5

For a, b, CO₂⁽¹⁾ and CO₂⁽²⁾, see Table 1.

The chloroplast pattern of ABA-treated tissues is similar to that of old tissues; groups I and III remain. After treatment with ABA, two important populations of chloroplasts appear in the 51 and 57% sucrose zones, in an identical pattern as for 110 day-old tissues. ABA accelerates explant aging and produces the typical chloroplast pattern of old tissues. With 10^{-4} M, "senescence" is set, almost all groups of chloroplasts are touched. Those of group III are destroyed, and the tissues become necrotic.

Conclusion

To explain the present data, two complementary hypotheses must be briefly presented. ABA may first have a direct action on chloroplast differentiation—as previously reported (7)—in carrot tissues cultivated in vitro, causing thereby an acceleration of tissue maturation. But ABA could also control the metabolism—as already discussed (12)—of the carrot tissues cultured in vitro, inducing some aging processes which could very well explain a rapid increase in the chloroplast content, usually higher in senescent tissues.

On the other hand, whichever type of treatment is applied to the tissues (pre-treatment or continuous application of ABA), it always results in an inhibition of the Ru-DP carboxylase of chloroplasts isolated from the carrot tissues cultivated in vitro and a strong decrease in the ¹⁴CO₂ fixation as well.

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