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NCC MALONYLTRANSFERASE CATALYSES THE FINAL STEP OF CHLOROPHYLL BREAKDOWN IN RAPE (*BRASSICA NAPUS*)

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Key Word Index—*Brassica napus*; Brassicaceae; chlorophyll breakdown; catabolites; malonyltransferase; senescence.

Abstract—Of the three final products of chlorophyll breakdown that in senescing cotyledons of oilseed rape are accumulated progressively, the nonfluorescent *Bn*-NCC-1 is the most abundant catabolite. It represents the malonylester of the minor catabolite *Bn*-NCC-3. The *in vitro* malonylation of *Bn*-NCC-3 into *Bn*-NCC-1 was investigated. Extracts from senescent as well as from presenescent cotyledons contained corresponding activities in the presence of malonyl-coenzyme A as the co-substrate. Malonyltransferase activity exhibited pH- and activation optima at 8 and 34°, respectively, and it was saturable with an apparent Michaelis constant of 58 μM for *Bn*-NCC-3. The partially purified enzyme recognized chlorophyll catabolites as substrate specifically, provided that they had a free hydroxyl group in the ethyl side chain of pyrrole B.

INTRODUCTION

The final products of chlorophyll (Chl) breakdown in senescent leaves have been identified as nonfluorescent tetrapyrrolic derivatives of phaeophorbide (Phaeide) *a*. These NCCs (nonfluorescent Chl catabolites) have a common basic structure of 1-oxo-19-formyl-bilanes that are modified in different side chains [1–5]. Thus, in rape, three NCCs (*Bn*-NCC-1, 2, 3; see [6] for nomenclature of Chl catabolites) have been identified which account for the total amount of Chl *a* and *b* present in mature cotyledons [7]. Whereas in *Bn*-NCC-3 the ethyl side chain of pyrrole B is merely hydroxylated, in *Bn*-NCC-1 it is esterified with malonic acid and in *Bn*-NCC-2 it is β-glucosylated [5] (Fig. 1). In that respect NCCs resemble secondary compounds such as anthocyanins which in many cases are also malonylated [8]. NCCs are deposited within the central vacuole of senescent leaves by the activity of a primary active carrier located at the tonoplast [9].

Of the reactions responsible for the breakdown of Chl to NCCs only the early steps have so far been partially characterized. The sequence of enzymic steps comprises (i) dephytylation of Chl to chlorophyllide (Chlide) by chlorophyllase, (ii) removal of the central Mg-atom by Mg-dechelatase, (iii) oxygenolytic cleavage of Phaeide *a* by Phaeide *a* oxygenase to a red Chl catabolite (RCC) followed by (iv) reduction of RCC to a primary fluorescent catabolite (pFCC) catalysed by RCC reductase [10] (Fig. 1). The primary FCC is subsequently metabolized to NCCs by additional steps that have not yet been studied in detail. Since the structure of pFCC of rape has been elucidated recently [11], the reactions responsible for the transformation to *Bn*-NCC-3 can be predicted to include hydroxylation at $C8^2$, tautomerization in pyrrole D and hydrolysis of the methylester at $C13^2$. As intermediary NCCs have not yet been discovered, no information about the sequence of these reactions is available.

In rape cotyledons a late if not final step results either in the esterification of the $C8^2$-hydroxyl group with malonic acid or in the glucosylation. Such a final reaction, the malonylation of *Bn*-NCC-3 to *Bn*-NCC-1, has now been demonstrated to be catalysed by a specific transferase present in rape cotyledons.

RESULTS AND DISCUSSION

Protein extracts from senescent rape cotyledons prepared at pH 8.0 were successfully employed for the synthesis of *Bn*-NCC-1 from *Bn*-NCC-3 in the presence of malonyl-CoA (Table 1). *Bn*-NCC-1 was identified by its retention time on reverse-phase HPLC and by its typical absorption spectrum with a maximum at 316 nm [5]. No reaction product occurred in the absence of either of the components present in the complete assay nor when a heat-denatured protein extract was employed. These results demonstrate that rape cotyledons contain a protein which catalyses the transmalonylation from malonyl-CoA to a Chl cat-

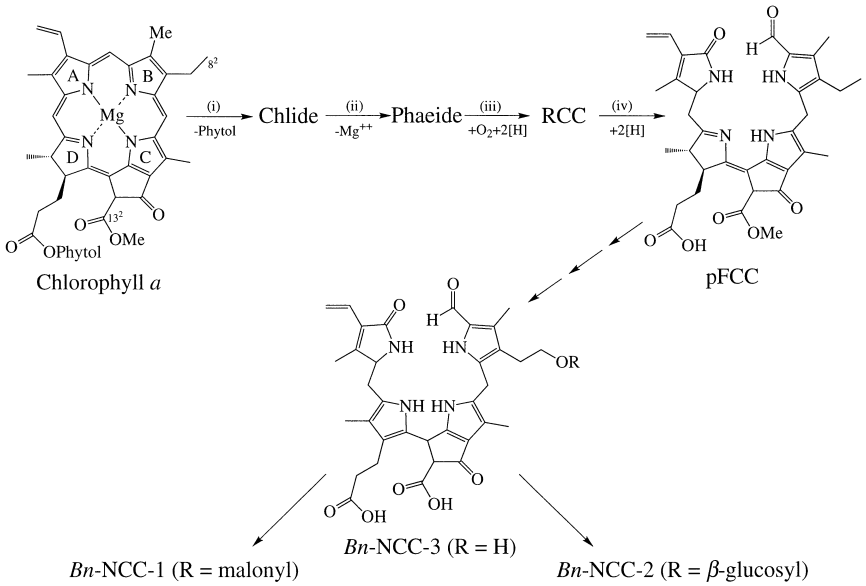

Fig. 1. Pathway of Chl breakdown in rape. The enzymes catalysing the formation of pFCC from Chl *a* are (i) chlorophyllase, (ii) Mg-dechelatase, (iii) Phaeide *a* oxygenase and (iv) RCC reductase. The downstream steps from pFCC to the NCCs are as yet unknown.

Table 1. NCC malonyltransferase activity in crude protein extracts from rape. The standard assay (control) contained 1 mM malonyl-CoA, 0.1 mM *Bn*-NCC-3 and 100 μg protein from senescent rape cotyledons

	Specific activities pkat (% activity)	
Assay conditions	mg^{-1} protein	g^{-1} fr. wt
Control (standard assay mixture)	2.9 (100)	2.20 (100)
—Malonyl-CoA	0.0 (0)	0.00 (0)
—*Bn*-NCC-3	0.2 (6)	0.13 (6)
—Protein extract	0.0 (0)	0.00 (0)
Boiled protein	0.0 (0)	0.00 (0)
Protein extracted at pH 6.0	1.0 (35)	0.53 (24)
Protein from presenescent tissue	1.1 (38)	1.88 (85)

abolite, *Bn*-NCC-3. This enzyme is tentatively named NCC malonyltransferase.

The reaction proceeded linearly for at least 40 min and exhibited a pH optimum of about 8 (data not shown) coinciding with the observation that NCC malonyltransferase activity of senescent rape cotyledons was particularly high if extracted at slightly alkaline pH; only about a third of these activities were present in extracts prepared at pH 6.0 (Table 1). Like other malonyltransferases [12, 13] NCC malonyltransferase had a comparatively high optimal activity at 34°. As the enzyme was rather unstable at 34°, standard incubations were performed at 30°. Extractions of rape cotyledons at different stages of development revealed that the specific activity of the malonyltransferase correlated with Chl breakdown in the senescent tissue (Table 1); referred to fresh weight the activities were similar in mature and senescent cotyledons.

NCC malonyltransferase activity was partially purified about 25-fold by fractionated precipitation with $(NH_4)_2SO_4$ (50–70% saturation) and subsequent chromatography on MonoQ (Table 2). Formation of *Bn*-NCC-1 exhibited saturation kinetics with an apparent K_m of 58 μM for *Bn*-NCC-3 (Fig. 2). The partially purified enzyme recognized NCCs from different sources, such as *Hv*-NCC-1 (barley) [1] or the major Chl catabolite from *Cercidiphyllum japonicum* [4] as substrates for esterification with malonic acid (Table 3). Both of these NCCs are hydroxylated at $C8^2$ but in contrast to *Bn*-NCC-3 they have a methylated carboxyl group at $C13^2$; in *Hv*-NCC-1, the vinyl side chain of ring A is modified into a dihydroxyvinl group. In contrast to these NCCs, *Nr*-NCC-1, the major cat-

Table 2. Partial purification of NCC malonyltransferase from senescent rape cotyledons

Purification step	Total activity pkat	Protein mg	Specific activity pkat mg^{-1}	Purification fold
Crude extract	200	38.1	5.25	—
$(NH_4)_2SO_4$ 50–70% saturation	176	8.9	19.8	3.8
MonoQ	149	1.14	130.7	24.9

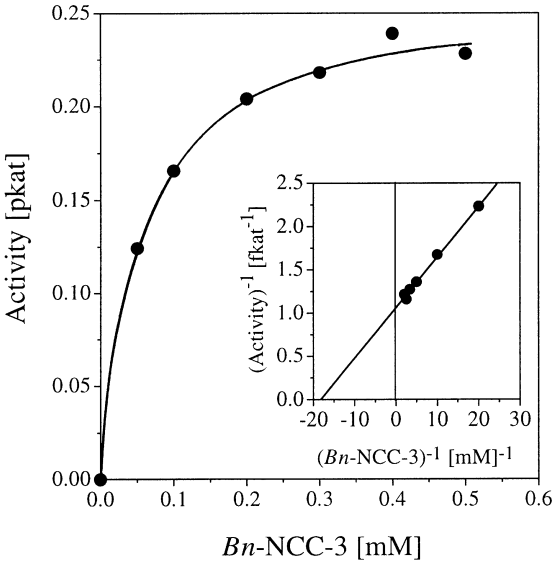

Fig. 2. Saturation kinetics of *Bn*-NCC-3 to *Bn*-NCC-1 malonylation as catalysed by NCC malonyltransferase. The Lineweaver–Burk plot (inset) indicates a K_m value of 58 μM.

abolite of *Nicotiana rustica* which is likely to be modified at the hydroxyl group at the $C8^2$ position (B. Kräutler, pers. comm.) was not accepted as a substrate, and it inhibited the malonylation of *Bn*-NCC-3 only slightly (Table 3). The addition to the standard assay of an excessive amount of 1-aminocyclopropane-1-carboxylic acid (ACC), the substrate for ACC malonyltransferase [12], failed to inhibit the conversion of *Bn*-NCC-3 to *Bn*-NCC-1 suggesting that two different transferases are responsible for the respective transmalonylation reactions.

A minor NCC of rape, *Bn*-NCC-2, represents the glucosylated analogue of *Bn*-NCC-1 [5]. It was tempting to explore whether the protein preparations from rape cotyledons containing NCC malonyltransferase activity contain also glucosyltransferase activity. However, incubations in the presence of *Bn*-NCC-3 and UDP-glucose, the common co-substrate of glucosylation reactions [14–16], failed to yield detectable amounts of *Bn*-NCC-2. That may be due to the presence of only a low activity of the respective transferase corresponding to the presence in senescent rape cotyledons of only trace amounts of *Bn*-NCC-2 compared to *Bn*-NCC-1 [6].

EXPERIMENTAL

Plant material

Seedlings of *Brassica napus* L. cv. Lirajet and of *Hordeum vulgare* L. cv. Express were grown in a growth chamber under a 12 h:12 h dark:light regime [17]. After 10 days, shoots were excised and, after placing them in flasks filled with tap water, senescence was induced by incubation in permanent darkness for 3 days (rape) and 6 days (barley), respectively. Plants of *N. rustica* were grown under greenhouse conditions until the end of the flowering period. For the isolation of the major Chl catabolite from *Cercidiphyllum japonicum* (*Cj*-NCC-1) autumn leaves were collected from a shrub in the local botanical garden.

Table 3. Substrate specificity of NCC malonyltransferase. The standard assay was performed with NCC malonyltransferase after chromatography on MonoQ (4 μg protein per assay). The malonylation products of the respective NCCs were calculated employing the absorption coefficient of *Bn*-NCC-1 [1]

Malonyl acceptors added	NCC malonyltransferase fkat (% activity)
Bn-NCC-3 (0.1 mM) (control)	583 (100)
Hv-NCC-1 (0.1 mM)	607 (104)
Cj-NCC-1 (0.1 mM)	543 (93)
Nr-NCC-1 (0.1 mM)	0 (0)
Bn-NCC-3 (0.1 mM) + *Nr*-NCC-1 (1 mM)	484 (83)
Bn-NCC-3 (0.1 mM) + ACC (1 mM)	581 (100)

Isolation of Chl catabolites

Senescent leaf material was homogenized in liquid N_2 and thawed in a soln of 25% (v/v) 25 mM *tris*-Mes pH 8.0 and 75% (v/v) MeOH (1 ml g^{-1} fr. wt). The mixture was centrifuged (15 min; 12,000 ***g***; 4°) and one vol. of MeOH was added to the supernatant. After a second centrifugation, the supernatant was evaporated by red. pres. Catabolites were separated by prep. HPLC [11] with 0.1 M K-Pi buffer pH 7–MeOH (13:7) for *Bn*-NCCs (rape) and *Hv*-NCC-1 (barley), and 0.1 M K-Pi buffer pH 7–MeOH (6:4) for *Nr*-NCC-1 (*N. rustica*) and the *C. japonicum* NCC.

Enzyme preparation

Cotyledons from either presenescent or senescent rape plants were blended in a Sorvall Omni-Mixer at maximal speed (3×10 s) in a buffer (2 ml g^{-1} tissue) containing 25 mM *tris*-Mes pH 8.0, 5 mM EDTA and 10 mM DTT and centrifuged (5 min; 24,000 ***g***). $(NH_4)_2SO_4$ was added to the supernatant first to a satn of 50% and, after removal of precipitated proteins by centrifugation (10 min; 38,000 ***g***), to a final concn of 70%. After centrifugation, the pellet containing malonyltransferase activity was dissolved in 25 mM *tris*-Mes pH 8 and desalted on Sephadex G25 fine. NCC malonyltransferase was further purified by anion exchange chromatography on MonoQ employing a 0 to 500 mM NaCl gradient in 25 mM *tris*-Mes pH 8. Active fractions were pooled and desalted on Sephadex G25 fine. For extraction at acidic pH, the *tris*-Mes buffer was replaced by 25 mM Mes-Imidazol (pH 6).

Malonyltransferase assay

The complete standard assay mixture contained, in a volume of 30 μl, 25 mM *tris*-Mes pH 8.0, 0.1 mM *Bn*-NCC-3, 1.0 mM malonyl-CoA and 20 μl either of the crude extracts or of fractions from the purification procedure (4 to 100 μg protein). After incubation at 30° for 30 min, the reaction was terminated by the addition of 30 μl MeOH. For characterization of different parameters of the malonyltransferase reaction, the standard assay was modified as indicated in tables and figures.

Identification of reaction products

MeOH extracts of assay mixtures were subjected to reverse-phase HPLC as described [7] employing an isocratic elution with 0.1 M K-Pi pH 7–MeOH (1:1) as solvent. The reaction product was identified by monitoring of absorption at 316 nm. Its identity as *Bn*-NCC-1 was confirmed by co-chromatography with authentic *Bn*-NCC-1 [2] and by online-monitoring of the absorption spectrum with a maximum at 316 nm, typical for NCCs [1, 5].

Protein determination

Protein was determined according ref. [18] with BSA as a standard protein.

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