Communication

How Plants Dispose of Chlorophyll Catabolites

DIRECTLY ENERGIZED UPTAKE OF TETRAPYRROLIC BREAKDOWN PRODUCTS INTO ISOLATED VACUOLES*

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During the yellowing of leaves the porphyrin moiety of chlorophyll is cleaved into colorless linear tetrapyrrolic catabolites, which eventually are deposited in the central vacuoles of mesophyll cells. In senescent cotyledons of rape, Brassica napus, three nonfluorescent chlorophyll catabolites (NCCs), accounting for practically all the chlorophyll broken down, were found to be located in the vacuoles (vacuoplasts) prepared from protoplasts. Transport of catabolites across the tonoplast was studied with vacuoles isolated from barley mesophyll protoplasts in conjunction with a radiolabeled pyrophosphate (NCC). The transport was independent of Mg2ATP and it followed saturation kinetics with a K_m of approximately 100 μM. Although the hydrolysis of ATP was required, transport was apparently independent of the vacuolar proton pumps: accumulation of the NCC occurred both in the presence of the H^+-ATPase inhibitor bafilomycin and after destroying the ΔpH between the vacuolar sap and the medium. ATP could be replaced by GTP or UTP, and the transport was inhibited in the presence of vanadate. Chlorophyll catabolites isolated from senescent barley leaves competed with the rape-specific substrate for uptake into the vacuoles. Compounds such as the glutathione conjugate of N-ethylmaleimide and taurocholate, which are known to be transported across the tonoplast in a primary active mode, did not significantly inhibit uptake of Bn-NCC-1. Although the heme catabolites biliverdin and bilirubin inhibited the uptake of the NCC, this effect is caused by unspecified binding to the vacuolar membrane rather than to the specific inhibition of carrier-mediated transport. Taken together, the results demonstrate that barley mesophyll vacuoles are constitutively equipped with a directly energized carrier that transports tetrapyrrolic catabolites of chlorophyll into the vacuole.

When leaves senesce and turn yellow, transition of mesophyll chloroplasts into gerontoplasts takes place. This type of plastid differentiation is characterized by the extensive loss of stroma proteins and thylakoid membranes so that fully developed gerontoplasts consist of a still intact plastid envelope enclosing barely more than a number of large plastoglobuli, which contain undigested or undigestible lipophilic materials (1). Gerontoplasts are competent for disassembly of thylakoidal pigment-protein complexes and, particularly, for the breakdown of chlorophyll (Chl) into phyto1, Mg2+, and water-soluble cleavage products of the porphyrin moiety. Whereas phyto1, still largely in esterified form, is accumulated in the plastoglobuli (2), catabolites of Chl-porphyrin are exported from developing gerontoplasts (3) and are, eventually, accumulated in the vacuoles of senescent barley mesophyll cells (4–6). Radiolabeling of Chl in the pyrrole units during greening of barley primary leaves and subsequent tracing of catabolites during senescence has shown that export of catabolites to other parts of the plant does not take place. Instead, the labeled catabolites remained sequestered in the vacuoles until the end of the senescence period (7).

Four catabolites, one from barley primary leaves and three from rape cotyledons, have so far been purified and analyzed with regard to chemical structure (8–11). All of them represent linear tetrapyrroles derived from phophorbide a through the oxidative cleavage of the porphyrin macrocycle in 4/5 mesoporation between pyrroles A and B (Fig. 1). The three NCCs of rape represent a family of related tetrapyrroles, with Bn-NCC-3 having the basic structure featuring a hydroxylated ethyl side chain in pyrrole B; in Bn-NCC-1 this hydroxyl group is esterified with malonic acid, whereas in Bn-NCC-2 it is glucosylated (11). These linear tetrapyrroles appear to represent the final breakdown products of Chl-porphyrin.

The primary product of cleavage of phophorbide a is characterized by its blue fluorescence (12–14). This fluorescent Chl catabolite (FCC) appears to be common in all plant species and, within gerontoplasts, seems to be modified to yield other FCCs with higher polarities. After ATP-dependent export from gerontoplasts (3) these catabolites are probably modified in the cytoplasm of the cell and eventually transported across the tonoplast as indicated by the localization in isolated barley mesophyll vacuoles of an FCC (Hv-FCC-2) together with an NCC (Hv-NCC-1) (5, 6). Results of experiments with isolated vacuoles reported here indicate that a specific carrier in the tonoplast is responsible for the final disposal of Chl catabolites in the cell sap.

MATERIALS AND METHODS

Cultivation of Plants—Seedlings of barley, Hordeum vulgare L. cv. Baraka and Express, and of oilseed rape, Brassica napus L. cv. Arabella, were grown under conditions described previously (14).

Preparation of Vacuoles—Primary barley leaves of the cultivar Baraka were harvested on day 10 after sowing and employed for the preparation of mesophyll protoplasts and of intact vacuoles (15).

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The abbreviations used are: Chl, chlorophyll; FCC, fluorescent chlorophyll catabolite; NCC, nonfluorescent chlorophyll catabolite; HPLC, high performance liquid chromatography.
order to avoid unspecific binding to the tonoplast of Chl catabolites during uptake experiments, vacuoles were incubated in the presence of 0.15 mM [14C]Hv-NCC-1 for 5 min prior to the last purification step. Contamination of isolated vacuoles (yield ~30%) with other cell constituents, e.g., cytosol, mitochondria, endoplasmic reticulum, and plastids, was routinely measured by marker enzyme activities (16) and was less then 1% for each marker enzyme. Protoplasts were prepared from senescent rape cotyledons and fractionated into vacuoplasts and mini-protoplasts as described in Ref. 17.

Isolation of Catabolites from Barley Leaves—The isolation of Hv-NCC-1 (RP-14 according to an earlier terminology) followed a previously described procedure (9). The absorption coefficient at 315 nm (9) was employed for determination of concentrations of the purified catabolite as well as of the other purified NCCs and FCCs (see below). For the isolation of Hv-FCC-2, excised leaves of the cultivar Express were loaded with 70 µg of [14C]Bn-NCC-1 Citrate Methylamine and further additions as indicated. Uptake experiments were started by the addition of 30 µl of vacuole suspension. After gentle mixing, the incubation mixture was overlayed with 200 µl of silicone oil AR 200 (Fluka) and 60 µl of water. For termination, the intact vacuoles were displaced through the silicone oil layer by centrifugation at 10,000 × g for 15 s. The radioactivity (14C and 3H) in the top aqueous phase released from the osmotically lysed vacuoles was measured by liquid scintillation counting. Unless otherwise mentioned, uptake rates were calculated by subtracting the 14C taken up after 3 min from the 15-min value. Numbers of vacuoles were calculated by taking advantage of the rapid equilibration of 3H2O between medium and cell sap (107 vacuoles correspond to 160 µl).

RESULTS AND DISCUSSION

Justification of the Heterologous System—Uptake experiments were performed with vacuoles from barley leaves and a catabolite from rape cotyledons. There were two reasons for choosing this heterologous system. First, the purification of vacuoles from rape cotyledons was impossible, because their densities are similar to those of protoplasts. Second, the preparation of a 14C-labeled catabolite from rape cotyledons was
easy and efficient because Bn-NCC-1 is predominant (18) and can readily be separated from the two minor catabolites. In contrast, the only catabolite from barley with a known structure, Hv-NCC-1 accounts for only 10–20% of all catabolites (19), making radiolabeling on a large scale inefficient. The disadvantage of the heterologous system was overcome by challenging the transport of the catabolite into barley vacuoles with two barley catabolites.

Localization of Rape Catabolites in Rape Vacuoles—Since the vacuolar location of Chl catabolites had been demonstrated only in senescent barley leaves (4–6), it was necessary to define the subcellular locations of these metabolites in rape. Compartmentalization analysis was performed with vacuoplasts, because we did not succeed in purifying intact vacuoles on a large scale. Vacuoplasts consist mainly of vacuoles surrounded by plasma-lemma that trap a small proportion of the cytosol. As shown in Table I, the three catabolites Bn-NCC-1, -2, and -3, which account for practically all the chlorophyll broken down (18), were recovered in the soluble fraction of vacuoplasts.

Uptake of Bn-NCC-1 into Isolated Barley Vacuoles—Upon incubation of isolated barley vacuoles in the presence of 14C-labeled Bn-NCC-1, a time-dependent accumulation of 14C in vacuoles was observed. Uptake proceeded linearly for up to 30 min, was strictly dependent on the presence of MgATP in the incubation medium (Fig. 2; Table II), and followed saturation kinetics with a K_m value of 110 μM (Fig. 3). These results suggest that the tonoplast of barley vacuoles contain a carrier that recognizes a rape-specific Chl catabolite. Fig. 3 also shows that the concentration in the standard assay mixture of 0.3 mM Bn-NCC-1 was sufficient for the saturation of transport. To save precious substrate, in some experiments the catabolite was also employed at a concentration of only 150 μM (see results presented in Table IV).

Tetrapyrrolic Chl catabolites have so far been detected exclusively in senescent leaves. Rather unexpectedly, vacuoles from mature leaves turned out to be as competent for the uptake of catabolites as were vacuoles prepared from senescent leaves (data not shown). Hence, the carrier in question seems to represent a constitutive component of the barley mesophyll tonoplast.

Energization of Transport—The strict dependence of uptake on MgATP suggested that the carrier of tetrapyrrolic Chl catabolites may be energized directly rather than through a ΔpH between cell sap and medium. Uptake driven by GTP or UTP as well as inhibition in the presence of vanadate (Table II) were also typical for primary active transport as observed in other instances (20, 21). Indeed, bafilomycin, an inhibitor of V-H^+-ATPase, did not inhibit catabolite uptake, whereas the transport of citrate was reduced to the −MgATP level. Abolishment of ΔpH in the presence of (NH_4)_2SO_4 also had no effect on the transport of Bn-NCC-1 in contrast to methylamine (Table II). Uptake of Bn-NCC-1 into isolated vacuoles resulted in net accumulation as demonstrated by significantly higher concentrations in the reisolated vacuoles than in the incubation medium; net accumulation was affected neither by bafilomycin nor by (NH_4)_2SO_4 (Table III). We conclude, therefore, that the catabolite carrier in the tonoplast functions in the primary active mode and resembles the carriers responsible for the deposition of glutathione S-conjugates (20) or bile acids (21) in the vacuole.

Specificity of Transport—Radiolabeled Bn-NCC-2, a minor rape catabolite, was successfully employed as an alternative substrate in uptake experiments (data not shown). Transport of Bn-NCC-1 into barley vacuoles was inhibited by two barley-specific catabolites, Hv-FCC-2 and Hv-NCC-1 (Table IV). These findings suggest that the carrier recognizes the basic structure of linear tetrapyrroles rather than some species-specific structural features of Chl catabolites. The determination of accurate K_i values for transport of barley catabolites would have been desirable, but it was impossible to isolate the amounts required for this purpose. However, estimations using the Michaelis-Menten kinetics reveal a K_i of about 60 and 370 μM for Hv-FCC-2 and Hv-NCC-1, respectively. The relatively weak inhibition of the NCC compared with the FCC, therefore, raises the possibility that the latter is the form transported across the tonoplast in vivo and that conversion to the NCCs occurs within the vacuole. An alternate possibility is that cytoplasmic proteins with a high affinity for NCCs bind and present the substrate to the transporter.

Whether or not the carrier is able to distinguish between linear tetrapyrroles originating from Chl and heme, respectively, was tested by measuring the uptake of Bn-NCC-1 in the presence of biliverdin or bilirubin. Both bile pigments, particularly biliverdin, caused marked inhibitions of NCC uptake (Table IV). Vacuoles reisolated after exposure to biliverdin contained large amounts of this heme catabolite. However, the accumulation turned out to be independent of the presence of MgATP and seems to be caused by binding to, rather than by

![Fig. 3. Saturation kinetics of Bn-NCC-1 uptake into isolated barley vacuoles. The Lineweaver-Burk plot (inset) indicates a K_m value of 110 μM. The incubation mixtures were supplemented with 3 mM MgATP.](image)

### Table III

| Treatment                  | Concentration of Bn-NCC-1 μM | Medium, initial | Internal (vacuole) |
|----------------------------|-------------------------------|----------------|-------------------|
|                            |                               | Experiment 1   | Experiment 2      |
|                            |                               | 30 min         | 50 min            | 30 min         | 50 min         |
| Control                    | 75                            | 198 ± 3        | 264 ± 26          | 217 ± 14       | 305 ± 15       |
| (NH_4)_2SO_4 (2.5 mM)      | 75                            | 183 ± 9        | 289 ± 10          | 207 ± 27       | 332 ± 18       |
| Bafilomycin (0.1 μM)       | 75                            | 171 ± 9        | 293 ± 4           | 225 ± 17       | 311 ± 8        |

Vacuoles were incubated for 30 and 50 min, respectively, in the presence of 75 μM [14C]Bn-NCC-1, 6 mM MgATP, and inhibitors as indicated. Vacuolar concentrations were calculated from the ratio of H_2O and [14C]Bn-NCC-1 in the medium and in the vacuolar fraction.
transport across, the tonoplast (data not shown). It is also possible that the heme catabolites compete with Chl catabolites for the binding site of the carrier without being translocated, or translocated very slowly, across the membrane.

Compounds known to be transported by directly energized carriers of the barley tonoplast, taurocholate, glycocholate, and the glutathione S-conjugates of N-ethylmaleimide and decane (20, 21), at saturating concentrations of the respective substrates, had only weak effects on the uptake of Bn-NCC-1 (Table IV). Hence, the tonoplast appears to be equipped with distinct carriers for Chl catabolites, glutathione S-conjugates and bile salts, respectively.

We were interested whether chlorophyll catabolites are also recognized by an organism where these substrates do not occur. Using yeast vacuole vesicles, we could not detect any transport activity (data not shown), in contrast to glutathione S-conjugates, which are transported across the vacuolar membrane in a primary active mode in yeast (22). This result indicates that plants may contain a specific transporter for chlorophyll catabolites that is not present in non-chlorophyllous organisms.

Conclusions—Directly energized transport has originally been demonstrated in human cancer cells, where overproduction of the multiple drug resistance protein conferred resistance to a variety of drugs (23). Multiple drug resistance proteins have domains that resemble the ATP-binding domain (ATP-binding cassette) of ABC transporters. These carriers are considered to play an important role in cellular detoxification (23). In yeast, more than 10 genes of this family have been identified (24), but the substrates recognized by the corresponding transporters are largely unknown.

In plant cells, directly energized transport into vacuoles has originally been discovered to be responsible for storage "export" of glutathione S-conjugates and bile acids (20, 21). The present report on the vacuolar disposal of Chl catabolites extends the range of this type of carriers to endogenously produced compounds. In this respect, the catabolite carrier described in this manuscript may not be unique since, recently, indirect evidence in favor of primary active transport into the vacuole of an glutathione S-conjugated anthocyanin has been reported (25). Hence, directly energized carriers in the tonoplast may be engaged in the disposal of a variety of endogenous secondary metabolites, including Chl catabolites.

The sequestration of secondary compounds in vacuoles must be conceived as detoxification of the cytoplasm (26). Due to its photodynamic nature, Chl is a toxic compound. Its detoxification through opening of the porphyrin macrocycle takes place in the senescent chloroplasts. It is, therefore, not easily understood why the abolishment of photodynamic behavior is followed by further metabolism and eventual disposal of linear tetrapyrroles in the cell sap. It is possible that these Chl catabolites are still toxic for hitherto unknown reasons. In any case, the existence of an elaborate and energy-consuming catabolic system suggests that the deposition of Chl catabolites in the vacuole may be necessary for senescence to take place in viable mesophyll cells.

### TABLE IV

| Treatment          | Transport of Bn-NCC-1 | % of control |
|--------------------|-----------------------|--------------|
| Control            | 100                   | 100          |
| Bn-NCC-1 (150 μM)  | 89                    | 89           |
| (1 mM)             | 42                    | 42           |
| Bn-FCC-2 (330 μM)  | 46                    | 46           |
| (150 μM)           | 44                    | 44           |
| Biliverdin (70 μM) | 23                    | 23           |
| (150 μM)           | 6                     | 6            |
| Taurocholate (300 μM) | 82                  | 82           |
| Glycocholate (300 μM) | 82                  | 82           |
| NEM-GS (1 mM)      | 99                    | 99           |
| Decyl-GS (200 μM)  | 64                    | 64           |

* Concentration of Bn-NCC-1 in the assay: 300 μM.

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