A detailed comparison of citrate uptake into the vacuole-like lutoids of rubber tree (Hevea brasiliensis Muell. Arg.) and of malate and citrate transport into barley (Hordeum vulgare L.) vacuoles revealed very similar transport specificities. In order to identify proteins mediating the transport, two photoreactive analogues (N-(2-hydroxy-5-azido)-diazo-N,3,5-benzenedicarboxylic acid and 5-azidoisophthalic acid) of malate/citrate were synthesized and found to efficiently inhibit citrate uptake into barley vacuoles (Ki = 18 μM) and Hevea lutoid vesicles (Ki = 27 μM). In vacuoles from both plant species, these photoreactive probes specifically labeled a single protein with a molecular mass of 23.6 kDa. This citrate binding protein (CBP) was purified to homogeneity from Hevea lutoids, and amino acid sequences were determined for NH2-terminal and tryptic peptides. Using degenerate oligonucleotides of the NH2-terminal sequence, a cDNA coding for the CBP protein of Hevea was isolated. The cDNA codes for a precursor protein of 238 amino acids, containing an NH2-terminal 31-amino acid signal sequence for endoplasmic reticulum targeting, a prerequisite for vacuolar localization. The mature CBP does not show significant sequence similarities to any known primary protein structure and thus represents a member of a novel class of proteins.

Malate and citrate play central roles in plant metabolism as intermediates of the TCA cycle and of CO2 fixation, as components of pH homeostasis and as the transport forms of reduc-
tive inhibitors (10–12). Specific inhibition of the ATPase indicated that malate transport occurs by a secondary active transport mechanism (13). A detailed comparison of the uptake of citrate and malate into barley vacuoles showed that the characteristics of uptake are very similar and this suggested that malate and citrate are transported by the same transport system across barley tonoplasts (14). However, in order to determine the substrate specificity in detail and, furthermore, to understand the regulation of malate and citrate transport into vacuoles, the identification of the protein(s) mediating malate and citrate uptake is required. The malate/citrate carrier of barley tonoplasts (15) and the malate transporter of Kalanchee daigremontiana vacuoles (16) have previously been functionally reconstituted and partially purified. The reconstituted carriers showed properties similar to those described for the carrier of intact vacuoles. However, further attempts to purify and identify the proteins mediating this transport activity were unsuccessful to date, due to limitations in the availability of tonoplast material and to the lack of methods for their detection.

In this study, we have compared citrate uptake into barley vacuoles and Hevea lutoid vesicles and were able to show that transport is efficiently inhibited by two photoreactive dicarboxylic acids. These photoaffinity probes specifically label a 23.6-kDa protein in both plant systems. We describe the isolation of the citrate binding protein of Hevea, the isolation of the corresponding cDNA and its gene, as well as the identification of homologous genes in barley.

MATERIALS AND METHODS
Isolation of Intact Barley Mesophyll Vacuoles—Barley (Hordeum vulgare L. cv Gerbel) mesophyll vacuoles were isolated and purified as described (14). Preparation of Lutoid Vesicles of Hevea—Latex was collected from trees of H. brasiliensis Muell. Arg., clone RRIM600, grown under natural conditions at the Rubber Research Center in Kuala Lumpur, Malaysia. Lutoids were sedimented by centrifugation at 4 °C. The pellet (or bottom fraction) which constituted the crude lutoid fraction was frozen in dry ice/alcohol immediately after separation from the centrifuged latex and kept on dry ice until use.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBankTM (¶) EMBL Data Bank with accession number(s) X89855.
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PHOTOAFFINITY LABELING, PURIFICATION, AND CLONING OF THE CORRESPONDING GENE*

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Aliquots of frozen Hevea lutoid fractions were thawed on ice in medium A (300 mM mannitol, 25 mM Mes, 125 mM Mops, 25 mM Hepes, adjusted to pH 6.0 with Tris base (6)). The suspension was homogenized on ice using a glass homogenizer (1 g bottom fraction per 10 ml of medium) and vesicles were sedimented by centrifugation at 10,000 × g for 15 min at 4 °C. The pellet was resuspended and centrifuged twice under identical conditions. To avoid contamination of lutoid membrane proteins by Triton X-100, vesicles were eluted with a citrate buffer at pH 2.8.

Purification of the CBP of Hevea Lutoid Vesicles—Solubilized lutoid membrane proteins (see functional reconstitution) were separated on an equilibrated (1% Triton X-100, 20 mM Tricine, pH 7.5) Sephadex G-75 column (superfine, Pharmacia; bed volume 45 ml, 30 cm × 1.5 cm). Proteins were eluted with a citrate buffer at pH 2.8. Fractions of 0.6 ml were collected, and aliquots of 100 μl were labeled. Fractions containing CBP were loaded on an equilibrated affinity column for the preparation of which 5-aminoisophthalic acid had been coupled to N-hydroxysuccinimide-activated agarose gel beads (Affi-Gel 15, Bio-Rad) according to the manufacturer’s instructions. The column was washed with 3 volumes of 20 mM Tricine, pH 7.5, 1% Triton X-100, and proteins were eluted with a citrate buffer at pH 2.8.

Sequence Determination of the ΗN2 Termi

Transport of malate and citrate was highly enriched by two purification steps, transport activity could not be confined to a single polypeptide (15). In a novel approach to identify putative carrier proteins for malate and citrate, photoactive analogues of the two acids were synthesized. Since isophthalate inhibited citrate and malate uptake very efficiently, derivatives of this compound were used as photoaffinity labels. Indeed, HABDA and AIPA were found to be potent competitive inhibitors of citrate transport and therefore seemed to be suitable for effi

After fluorography gels were detached from filter paper by soaking in 4% glycerol and stained with silver nitrate (19).
A probe could be prevented by addition of 10 mM citrate before and fluorography (Fig. 2). Since binding of the photoaffinity probe, the availability of barley putative malate and citrate transporters (15). However, for purifica-
citrate transport can still be measured in a reconstituted system (15). This purified protein fraction was irradiated in the presence of [3H]AIPA and in the presence of competing citrate. A specifically labeled protein with an apparent molecular mass of 27 kDa was identified after SDS-PAGE and fluorography (Fig. 2). Since binding of the photoaffinity probe could be prevented by addition of 10 mM citrate before irradiation, the labeled protein was a good candidate for a putative malate and citrate transporter. However, for purification of the labeled polypeptide and for more detailed analyses of binding of the photoaffinity probes, the availability of barley tonoplasts was limiting. Vacuoles from Hevea latex seemed to be an advantageous source of tonoplast material, since large amounts can be isolated with a minimum of preparative efforts. To demonstrate similarity to the described transport systems in barley mesophyll vacuoles, it was, however, necessary to characterize citrate transport specificity into lutoid vesicles in more detail.

Characterization of Citrate Transport into Hevea Lutoid Vesicles—Citrate transport into lutoids was stimulated by MgATP and the 

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characterization of the citrate uptake system of lutoid vesicles, the rates of citrate transport in the presence of various carboxylates and other anions were determined. Of the naturally occurring dicarboxylates tested, only malate and succinate inhibited citrate transport, while oxaloacetate had no significant effect (Table I, A). The monocarboxylate α-hydroxybutyrate did not affect citrate uptake either. Isocitrate weakly inhibited citrate uptake, whereas neither sulfate nor glutamate were inhibiting. The photoaffinity probe HABDA competed for citrate uptake into lutoid vesicles with a Ki of 27 μM. As shown for barley vacuoles, pyridoxal phosphate was a strong inhibitor for citrate uptake in Hevea lutoids as well (not shown). To demonstrate specific uptake, [14C]sorbitol was used as a control; uptake was slow and similar under all conditions (not shown; sorbitol: 1.5 nmol g of lutoids⁻¹ × min⁻¹; citrate: 42.3 nmol g of lutoids⁻¹ × min⁻¹). As the degree of inhibition of citrate uptake into lutoid vesicles was similar to that observed for citrate and malate transport into barley vacuoles (10, 14) (Table I, A), we conclude that the vacuolar citrate carriers of the two plant species have similar substrate specificities.

Labeling of Hevea Lutoid Vesicles—Hevea lutoid vesicles were irradiated in the presence of [3H]HABDA with or without competing citrate. As in barley tonoplasts, a polypeptide with an apparent molecular mass of 27 kDa was labeled, and citrate competed with the binding of the photoaffinity label (Fig. 3). Labeling with [3H]AIPA gave similar results (data not shown). Fluorography of control samples incubated for 5 min in the

![Structure of HABDA (A) and AIPA (B). The position of the tritium label is marked by an asterisk.](Image)

**Fig. 1.** Structure of HABDA (A) and AIPA (B). The position of the tritium label is marked by an asterisk.

| A | Citrate transport activity | B | Binding capacity of [3H]HABDA to CBP of lutoid vesicles |
|---|--------------------------|---|-------------------------------------------------------|
| ** competitors** | **Barley vacuoles** | **Lutoid vesicles** | **% of control** | **% of control** |
| α-Hydroxybutyrate | 86 ± 4 | 94 ± 9 | 110 ± 20 |
| L-Malate | 61 ± 2 | 41 ± 5 | 56 ± 2 |
| Succinate | 88 ± 1 | 56 ± 12 | 53 ± 3 |
| Oxaloacetate | 81 ± 5 | 94 ± 2 | 106 ± 3 |
| Citrate | 33 ± 4 | 25 ± 8 | 34 ± 10 |
| Isocitrate | 93 ± 7 | 77 ± 10 | 36 ± 9 |
| Isophthalate | 39 ± 5 | 50 ± 8 | 35 ± 5 |
| HABDA | 3 ± 2 | 5 ± 4 | 0 |
| AIPA | 6 ± 3 | ND* | ND |
| 1,2,3-Benzencarboxylate | 28 ± 5 | ND | 37 ± 9 |
| L-Glutamate | 98 ± 7 | 94 ± 5 | ND |
| Sulfate | 108 ± 8 | 108 ± 2 | 88 ± 4 |

*ND, not determined.
dark or under the same dimmed light conditions as used for the uptake experiments, did not reveal any labeled protein (not shown). Separation of proteins on polyacrylamide gels without urea resulted in a slightly lower apparent molecular mass of the labeled protein is tightly associated with the lutoid membrane, the CBP is probably not an integral membrane protein.

Inhibition of uptake experiments, did not reveal any labeled protein (not shown). Separation of proteins on polyacrylamide gels without urea resulted in a slightly lower apparent molecular mass of the labeled protein is tightly associated with the lutoid membrane, the CBP is probably not an integral membrane protein.

To determine the specificity of binding of the photoaffinity probe, di- and tricarboxylates and other anions were tested for specific labeling by $[^3$H]HABDA. For further purification of CBP, an affinity column using aminoisophthalate as ligand was used and proteins bound to the column were eluted with increasing citrate concentrations. Elution with 4 to 8 mM citrate resulted in highly purified CBP (Fig. 4).

Reconstitution of Citrate Transport Activity of Lutoids—Total lutoid membrane proteins reconstituted into liposomes showed a time-dependent citrate uptake which could be inhibited by an excess of citrate, malate, isophthalate, and pyridoxal phosphate, whereas no inhibition was observed by sulfate or oxaloacetate (Table II). Dissipation of the proton gradient (which was employed to energize uptake) by the two protonophores FCCP and CCCP, or by sonication of the liposomes after generation of the proton gradient resulted in a drastically reduced citrate transport activity. In contrast to the reconstituted malate or citrate carrier from barley tonoplasts (15) for which an imposed membrane potential was used as driving force for malate uptake, no uptake of citrate could be measured under these conditions with reconstituted lutoid proteins. During purification of CBP, fractions were tested for their ability to mediate citrate transport after functional reconstitution. No
citrate transport activity could be detected after separation on a Sephadex G-75 gel filtration column. However, when solubilized proteins of lutoid vesicles were directly loaded on isopthalate-Sepharose affinity columns, proteins eluting at citrate concentrations of 4–8 mM (this fraction contained the CBP) showed citrate transport properties similar to total lutoid membrane proteins when functionally reconstituted into liposomes (Table II). Citrate transport characteristics were as described for lutoid vesicles or total reconstituted lutoid membrane proteins, in that oxaloacetate did not reduce the rate of citrate uptake, whereas pyridoxal phosphate, citrate, and the two ionophores CCCP and FCCP caused significant reduction in citrate transport. However, additional purification steps resulted in very low uptake activities which did not allow us to determine specificities for the highly purified CBP.

Isolation of a cDNA Coding for CBP of Hevea—The purified protein fraction (Fig. 4) was used to determine the amino acid sequence of the NH₂ terminus of HcCBP and of peptides generated by digestion with trypsin (Table III). Degenerate oligonucleotides from the NH₂ terminus of CBP were used in a PCR with latex-specific cDNA from Hevea as template. The resulting isolated 54-bp PCR product was used as a probe to isolate a 227-bp fragment encoding the NH₂ terminus of the purified CBP from a latex-specific cDNA library. Since screening of several latex-specific cDNA libraries with the 227-bp fragment as probe did not result in additional cDNA clones encoding the COOH terminus of CBP, the gene was isolated by inverse PCR using nested primers and PstI-digested and subsequently circularized genomic DNA as template. This resulted in the isolation of a 1.4-kb genomic fragment. The S' end of the genomic PCR fragment was identical to the 3' end of the 227-bp cDNA fragment (Fig. 5) and contained an open reading frame including sequences located 3' of the EcoRI site terminating the 227-bp NH₂-terminal cDNA fragment. By using this genomic 1.4-kb PCR product as a probe to screen the cDNA library, a 630-bp cDNA fragment encoding the COOH-terminal moiety of CBP was isolated. As depicted in Fig. 5, the isolated NH₂- and COOH-terminal cDNA fragments represent clones of the same gene, adjacent to the EcoRI site. The genomic 1.4-kb PCR fragment covers the entire cDNA sequence starting from primer Hc6 at position 142 and contains only one intron of 174 bp at position 311. The first 5' ATG of the assembled CBP-cDNA sequence resides at position 29 (Fig. 6). At position 857 the first ATG of the assembled CBP-cDNA fragment covers the entire cDNA sequence starting from the EcoRI site. The genomic 1.4-kb PCR fragment, which links the two cDNA fragments at the EcoRI site, is shown at the bottom.

**Table III**

| Amino acid sequence of the NH₂ terminus and of tryptic peptides of HcCBP as determined by amino acid analysis |
|---------------------------------------------------------------|
| **NH₂ terminus** | **DPTGFTEPVLTEDNFVIQ** |
| **Tryptic peptides** | **LVVNDGGPAEH** |
|                   | **VNVHIKK** |
|                   | **VYNGLD** |
|                   | **ATALQ** |

**Fig. 5.** Schematic overview of cDNA and genomic clones encoding rubber tree CBP. NH₂-terminal (227 bp) and COOH-terminal (630 bp) cDNA fragments are shown in the upper part, the genomic 1.4-kb PCR fragment, which flank the two cDNA fragments at the EcoRI site, is shown at the bottom.

**Fig. 6.** Complete cDNA sequence and deduced amino acid sequence of HcCBP. Fragments were assembled as shown in Fig. 5. The NH₂-terminal amino acid sequence and tryptic peptides sequences determined by amino acid sequence analysis of the mature protein are underlined. Putative polyadenylation signals are typed bold.

32, contains 207 amino acids and has calculated molecular mass of 23.6 kDa.

A database search for related protein sequences revealed no significant similarities to known primary protein structures. Analysis of the hydrophobicity of the deduced amino acid sequence demonstrates that CBP is hydrophilic and contains only one hydrophobic stretch which might not be sufficient to act as a membrane spanning domain (Fig. 7B) (24). Predictions for the secondary structure indicate that no amphipathic helices large enough to span the lipid bilayer are likely to be present in CBP. The hydrophobic NH₂ terminus of the primary translation product cannot be used for integration into the membrane since the signal peptide is not present in the mature protein.

Homologous CBP Genes in Hevea and Barley—To determine the number of CBP encoding genes in Hevea, Southern blot analysis was performed under nonstringent conditions using the 630-bp COOH-terminal cDNA fragment as probe (Fig. 8A). Independently of the used restriction enzymes, only one or two bands are detectable on the blot. This simple pattern suggests that CBP is encoded by a single gene per haploid genome in Hevea. Interestingly, under comparable stringency the rubber...
The concentration of malate increases in C3 plants during light-dependent nitrate reduction, and in plants exhibiting CAM metabolism malic acid accumulates as a store for CO2 during the night. However, the concentration of malate in the cytosol remains rather constant and excess malate is transferred rapidly into the vacuole, where it accumulates to considerable levels (for review, see Ref. 1). Diurnal fluctuations of malate in C3 and CAM plants thus predominantly represent changes in vacuolar malate concentration and require malate transport across the tonoplast. Besides malate, citrate levels are subject to substantial day-night changes in CAM plants (25). Vacular malate and citrate transport have been shown in a variety of species and have been characterized at the biochemical level in barley vacuoles and tonoplast vesicles of CAM plants (10, 12). In barley vacuoles, citrate and malate appear to be transported by the same system (14). Purification of the proteins mediating the transport of malate and citrate has been limited due to the difficulties in obtaining tonoplast from barley vacuoles in sufficient quantities. Hevea luides represent an attractive source for the purification of tonoplast proteins since they can efficiently be isolated from latex sap. Previous investigations on citrate uptake into lutoid vesicles focused mainly on the energetics of transport (26). The detailed comparison presented here has now revealed that citrate transport into Hevea lutoid vesicles exhibits characteristics similar to those of malate and citrate uptake into barley vacuoles.

Similarity of Biochemical Properties of Citrate Transport—Malate, citrate, isophthalate and HABDA proved to be inhibitors of citrate transport in barley tonoplasts and Hevea lutoid vesicles. In contrast, neither α-hydroxybutyrate, oxaloacetate, glutamate, nor sulfate caused marked inhibition of uptake. However, differences between the vacuolar citrate transport activities of the two plant species were found upon reconstitution into liposomes. Even though substrate specificity and Km did not differ between vacuoles and lutoid vesicles, the respective driving forces for uptake did differ. Malate (15) and citrate uptake into proteoliposomes of solubilized and reconstituted barley tonoplast proteins was driven by a membrane potential and was strictly voltage-dependent. The exclusive role of Δψ as driving force for malate uptake has also been demonstrated for the reconstituted malate carrier of K. daigremontiana vacuoles (16), and voltage dependent channels involved in vacuolar malate transport have been described for CAM plants (27, 28). In contrast, uptake of citrate could not be measured unless a H+-gradient was generated as driving force (inside acidic). This would in fact support the exchange of citrate for H+ as proposed by Marin (5). Another explanation would be that an acidic environment inside the lutoids is required for efficient dissociation of citrate from the carrier. The use of photoaffinity probes strongly inhibiting citrate uptake nevertheless allowed the identification of apparently similar proteins.

Specificity of Binding of the Photoaffinity Label—Radiola-abeled photoreactive dicarboxylates specifically labeled a protein with a molecular mass of 23.6 kDa both in barley tonoplasts and in Hevea lutoid vesicles. With the exception of isocitrate, substrate specificity for binding of the photoaffinity probe to lutoid vesicles in the presence of competing anions was similar to inhibition of citrate uptake into these vesicles as well as barley vacuoles. Even though it was not possible to demonstrate citrate transport activity for the highly purified CBP, the data strongly suggest that CBP is involved in vacuolar citrate transport in both plant species. However, neither a citrate transporter from Salomonella typhimurium (29), the C4 dicarboxylate transporter from Rhizobium meliloti (30), nor the dicarboxylate transporter of the peribacteroid membrane of soybean nodules (31) could be labeled under the same conditions (not shown).

Sequence Analysis of CBP and Similarities with Other Proteins—Purification allowed the isolation of CBP and the subsequent cloning of the respective cDNA. A database search for related sequences revealed that CBP does not share significant homology with any known protein sequence. HbCBP therefore...
encodes a member of a new class of proteins. The deduced amino acid sequence contains a 31-amino acid signal peptide not present in the mature protein. This leader sequence is thought to be involved in targeting the protein to the endoplasmic reticulum based on its homology to endoplasmic reticulum targeting signals (22, 23). In contrast, integral membrane proteins such as the tonoplast intrinsic protein and the plasma membrane permeases for amino acids and ammonium do not seem to contain cleavable signal peptides for endoplasmic reticulum targeting (32–34). Because of its isolation from lutoids, CBP is expected to contain additional information for vacuolar targeting.

Analysis of its hydrophobicity indicates that CBP does not represent an integral membrane protein with multiple hydrophobic domains. This is in agreement with biochemical data showing that CBP is loosely associated with the lutoid membrane. In a parallel case, an affinity probe for sucrose allowed the identification of a hydrophilic 62-kDa sucrose binding protein (35) which is associated with the plasma membrane and appears to be involved in sucrose transport. The low hydrophobicity of CBP indicates that it does not represent the actual pore-forming subunit of the putative citrate transporter. However, binding of the photoaffinity probe and inhibition characteristics of citrate transport suggest that CBP may represent a peripheral subunit involved in substrate recognition. Of the few tonoplast proteins identified to date, the vacuolar ATPase is an example of a heteromultimeric complex (36). In Escherichia coli, amtA probably represents a cytoplasmic component of the ammonium transporter (37). Periplasmic proteins responsible for binding and subsequent transport of substrates (38) are required for the uptake of various solutes and provide further examples for transport not being mediated by a single polypeptide.

Conclusion—Photoaffinity labeling allowed the identification of a new protein which may represent a subunit of a vacuolar malate and citrate transporter. Further biochemical experiments, as well as the isolation of homologous genes and analyses of the function in transgenic plants are required to unambiguously define the function of CBP.

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