nature cell biology

Evidence for a protein transported through the secretory pathway *en route* to the higher plant chloroplast

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In contrast to animal and fungal cells, green plant cells contain one or multiple chloroplasts, the organelle(s) in which photosynthetic reactions take place. Chloroplasts are believed to have originated from an endosymbiotic event and contain DNA that codes for some of their proteins. Most chloroplast proteins are encoded by the nuclear genome and imported with the help of sorting signals that are intrinsic parts of the polypeptides. Here, we show that a chloroplast-located protein in higher plants takes an alternative route through the secretory pathway, and becomes *N*-glycosylated before entering the chloroplast.

Sorting of newly synthesized proteins to subcellular compartments is a fundamental process in all eukaryotic organisms. Chloroplasts are believed to have originated from an endosymbiotic event in which a prokaryotic cell with its own genome complement was engulfed by a eukaryotic host[1]. During evolution, most endosymbiont genes were transferred from the progenitor organelle to the host-cell nucleus[1,2]. Accordingly, the vast majority of chloroplast proteins are encoded by the nuclear genome and synthesized in precursor form on cytosolic ribosomes[3,4]. Chloroplast precursor proteins contain cleavable transit peptides, which direct them to the chloroplast in an organelle-specific way[4,5]. Translocation into chloroplasts occurs post-translationally and involves binding of precursor polypeptides to the Toc/Tic apparatus in the chloroplast envelope[4,5]. Recent studies of the chloroplast proteome have revealed the occurrence of many proteins without predicted transit peptides[6,7], indicating that targeting to chloroplasts may be more complex than was first expected[8].

In our studies on chloroplast-localized proteins, we identified an *Arabidopsis thaliana* expressed sequence tag (EST) (Z18493) that encodes an α-carbonic anhydrase (α-CA). The respective cDNA, which we denoted *CAH1*, contains a 1,046 base-pair open reading frame (ORF) that encodes a polypeptide of 284 amino acids. The CAH1 protein shares approximately 30% amino-acid sequence identity and 45% similarity with other α-CA sequences. It contains all 15 conserved catalytic and zinc binding residues that are typical for active α-CAs[9] (Fig. 1a, green and blue residues). Like secreted CAs, CAH1 has an extended amino-terminal sequence compared with the human CAs I, II and III (Fig. 1a, italics). TargetP analyses[10,11] predict that this sequence targets the protein to the endoplasmic reticulum (ER) and locates a potential signal peptidase cleavage site between amino acids 24 and 25 (ADA|Q, Fig. 1a, arrow). Stop codons in all three ORFs are found upstream of the putative initiator Met, ruling out the existence of an additional targeting sequence. To localize CAH1 within the cell, we performed immunolocalization analysis in *Arabidopsis* leaves. Unexpectedly, the results indicated that CAH1 is located exclusively in the chloroplast stroma (Fig. 1b, c, see Supplementary Information, Fig. S1a). To confirm this localization pattern, we analysed the presence of CAH1 in highly pure chloroplast and sub-plastid preparations (see Supplementary Information, Fig. S1b). CAH1 was enriched in intact chloroplasts and the stroma fraction, in a similar manner to the enrichment of the large subunit of Rubisco (see band above relative molecular mass 47,000), compared with total cell extracts (Fig. 1d). To exclude the possibility of contamination of the plastid fraction, we performed additional experiments. First, we tested the thermolysin susceptibility of CAH1. CAH1 was completely resistant to thermolysin treatment of intact chloroplasts, but was found to be susceptible after lysis of the chloroplasts (see Supplementary Information, Fig. S1c). All these results indicate that CAH1 is located in the stroma. As an independent verification of the CAH1 localization pattern, green fluorescent protein (GFP) was fused to the carboxyl terminus of full length CAH1. When transiently expressed in *Arabidopsis* protoplasts CAH1–GFP was targeted to the chloroplasts (Fig. 1e). Non-fused GFP protein (negative control) was distributed uniformly throughout the

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Received 29 August 2005; accepted 29 September 2005; published online: 13 November 2005; DOI: 10.1038/ncb1330

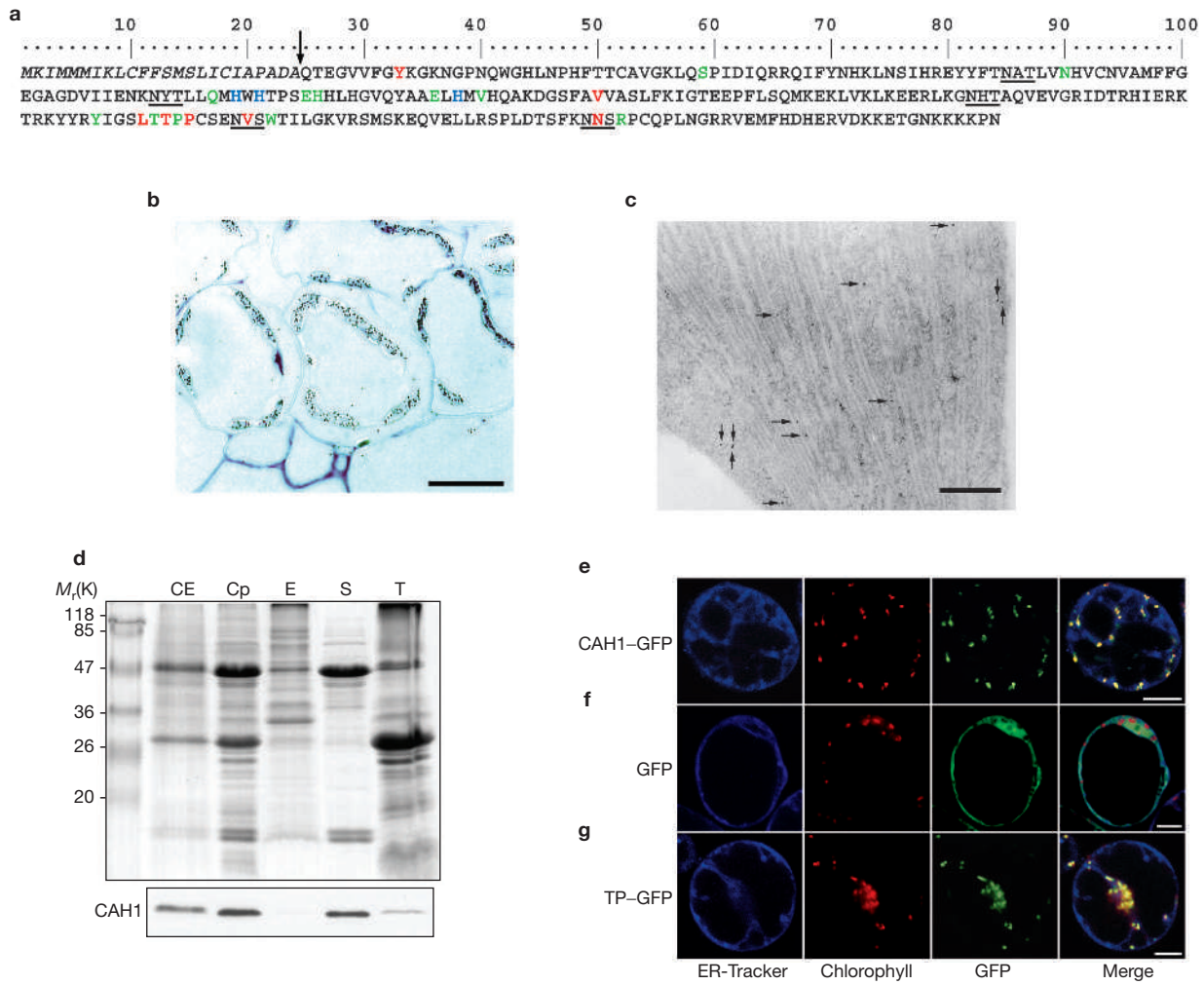

Figure 1 The deduced amino-acid sequence of CAH1 (**a**). Red-, blue- and green-coloured amino-acid residues correspond to 22 out of 36 putative active-site amino acids in α-type carbonic anhydrases (α-CAs). Blue histidine residues denote zinc binding ligands, which are conserved in all active α-CAs. Green residues are conserved in the active site of α-type CAs, whereas red residues are conserved but found outside the active site. The predicted signal peptide is shown in italics and the predicted processing site is indicated by an arrow. Underlined triplets indicate possible *N*-glycosylation sites. CAH1 is localized in the *Arabidopsis* chloroplast stroma. (**b**, **c**) Immunogold labelling of CAH1 in *Arabidopsis* leaves, indicating that this protein is located in the chloroplast stroma. Sections were examined under the light microscope (**b**), scale bar, 20 μm; or the electron microscope (**c**), the arrows indicate gold particles, scale bar, 100 nm. (**d**) Coomassie staining (top panel) and immunoblot with CAH1 antibodies of total leaf extracts and different chloroplast sub-fractions: CE, total leaf extract; Cp, total chloroplast extract; E, chloroplast envelope membranes; S, stroma; T, thylakoid membranes. K, relative molecular mass in thousands. (**e, f, g**) Transient expression of the green fluorescent protein (GFP)-tagged form of the *Arabidopsis* CAH1 protein in *Arabidopsis* cells under the 35S promoter. Scale bar, 10 μm (**e**). As subcellular localization controls, we used ER-Tracker (Molecular Probes) staining to assess the 35S–sGFP(S65T) plasmid (GFP) (**f**) and the 35S-TP–sGFP(S65T) plasmid (TP–GFP) containing the transit peptide (TP) sequence from RbcS fused to GFP. Scale bars, 10 μm (**f**, **g**).

cytosol and nucleus (Fig. 1f), whereas GFP that was fused to the transit peptide sequence of RbcS was targeted to chloroplasts (Fig. 1g). It therefore seems that the CAH1 sequence information is sufficient for chloroplast targeting of the fusion protein *in vivo*.

We next determined the functionality of the predicted CAH1 signal peptide (SP). The N-terminal 40 amino-acid residues of CAH1, containing the predicted ER SP, were fused to GFP harbouring an ER retention signal (KDEL) at the C terminus. When expressed in protoplasts, this fusion protein was retained in the ER (Fig. 2a), as shown by colocalization with a selective marker for the ER (ER-Tracker), indicating that the CAH1 SP is functional and sufficient for targeting the protein to the ER. This was confirmed by N-terminal sequencing of the gene product of this construct. The cleavage site was located between ADAQ/T, almost exactly at the site that was predicted by TargetP. In addition, when the full-length protein was fused to GFP containing a C-terminal KDEL sequence, the fusion protein was also retained in the ER. The GFP signal precisely overlapped with that of the ER-Tracker (Fig. 2b), as well as ER-targeted DsRed2-ER (see Supplementary Information, Fig. S1d). We next performed uptake studies both with isolated chloroplasts and ER-derived dog pancreas microsomes[12]. Intriguingly, intact pea chloroplasts were not able to take up or process the full-length CAH1 precursor or CAH1 lacking the SP (see Supplementary Information, Fig. S1e), indicating that translocation of CAH1 across the envelope membranes may not take place through the Toc/Tic apparatus. By contrast, efficient uptake, SP removal and glycosylation were observed in microsome preparations (Fig. 2c). The SP proved to be required for uptake of the protein into the microsomes, as a truncated CAH1 form lacking SP was not taken up into the ER, as shown by lack of glycosylation and sensitivity to externally

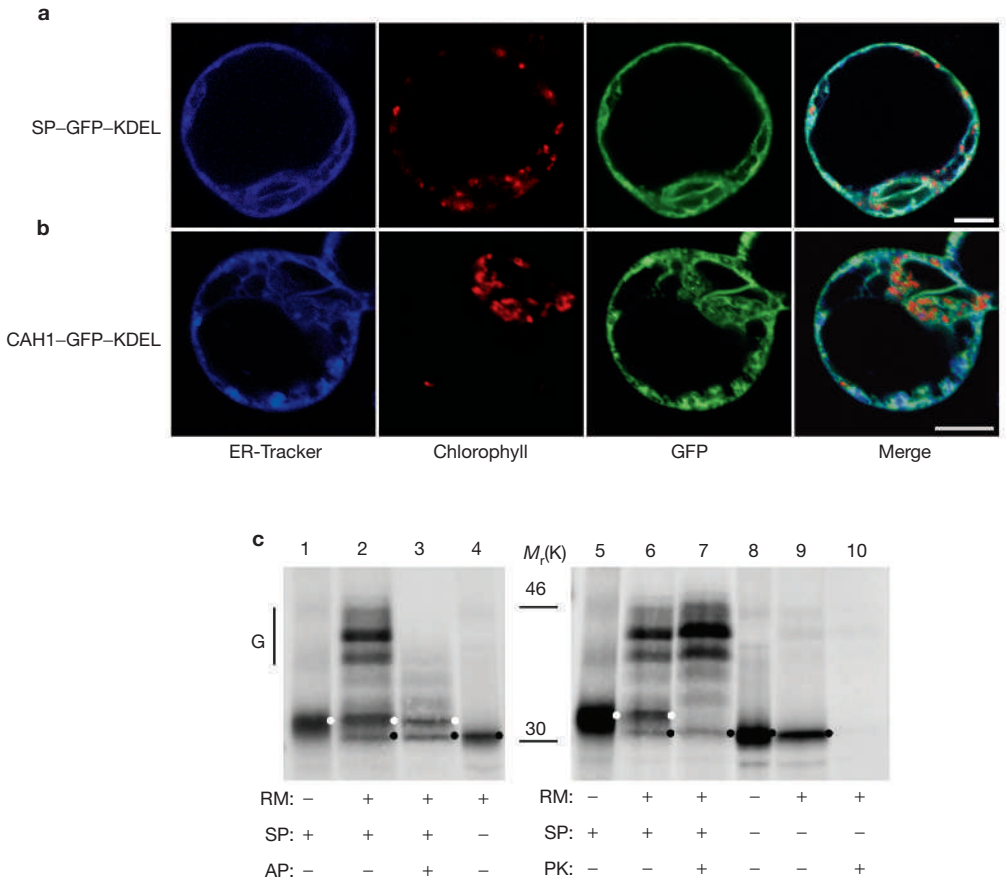

Figure 2 CAH1 is taken up into the endoplasmic reticulum and glycosylated. (**a**, **b**) Retention in the endoplasmic reticulum (ER) of ER retention signal (KDEL)-tagged CAH1–GFP (green fluorescent protein) fusion proteins, transiently expressed in *Arabidopsis* cells. Scale bar, 10 µm. As subcellular localization controls, cells were stained with a dye that is selective for the ER (ER-Tracker). (**a**) The first 40 amino-acid residues of CAH1, containing the signal peptide (SP) for the ER, were fused to the amino terminus of a KDEL-tagged GFP. (**b**) The full-length CAH1 was fused to the N-terminus of a KDEL-tagged GFP. (**c**) *In vitro* uptake into dog pancreas microsomes. CAH1 (lanes 1, 2, 3, 5, 6 and 7) and a truncated version lacking the signal peptide (SP) (lanes 4, 8, 9 and 10) were expressed *in vitro* in the absence (–) and presence (+) of rough dog pancreas microsomes (RM), either with no additions (lanes 1, 2, 4, 5, 6, 8 and 9), with addition of the competitive glycosylation inhibitor benzoyl-Asn-Leu-Thr-methylamide (AP) (lane 3) or with addition of protease (PK) (lanes 7 and 10). The precursor and the processed form of CAH1 are indicated by white and black dots, respectively. The wild-type CAH1 is taken up into the microsomes, glycosylated and the signal peptide cleaved off. The truncated form of CAH1, lacking the signal peptide, is not taken up into the microsomes. G, *N*-glycosylated isoforms.

added proteinase K (Fig. 2c, lanes 4, 8–10). With full-length CAH1, the SP is cleaved off after import, leading to a shift in mobility (Fig. 2c, lanes 3, 4). These findings demonstrate *in vitro* targeting of CAH1 to microsomes, but not chloroplasts, via its N-terminal SP.

The CAH1 protein has five predicted acceptor sites for *N*-linked glycosylation (Fig. 1a, underlined triplets), and products with relative molecular masses of 38,000, 41,000 and 44,000 are observed in addition to the CAH1 precursor and the unglycosylated, signal-peptidase processed form of the protein (Fig. 2c, lane 2). Addition of a competitive glycosylation inhibitor peptide prevents the appearance of the high-molecular-weight products (Fig. 2c, lane 3). The glycosylated and the unglycosylated signal-peptidase processed forms of the protein are resistant to externally added proteinase K (Fig. 2c, lane 7), and are therefore located in the lumen of microsomes. These findings indicate that CAH1 is not only taken up into the ER but is also glycosylated prior to being targeted to the chloroplast.

We addressed potential glycosylation of CAH1 in an independent experiment. CAH1–GFP and CAH1–GFP–KDEL proteins from transformed *Arabidopsis* protoplasts were immunoprecipitated with either anti-GFP or anti-CAH1 antibodies, and immunoblots were probed with the respective antibody that had not been used for immunoprecipitation (see Supplementary Information, Fig. S2a, b). As expected, both antibodies precipitated and detected a protein of the same size, demonstrating specificity of the CAH1 antibody. We then examined glycosylation of CAH1–GFP by probing the blots of these immunocomplexes with α(1,3)-fucose antibodies. Only the CAH1–GFP fusion was detected, whereas the construct carrying the KDEL ER retention signal did not show fucosylation (Fig. 3a). This result indicates that CAH1–GFP leaves the ER for the Golgi, as this is the organelle in which the fucose transferase is located[13], and ER retention of CAH1–GFP prevents this fucosylation.

We next examined whether glycosylated CAH1 accumulates in the chloroplast and whether this could explain the discrepancy between the apparent molecular mass of the mature protein that was isolated from chloroplasts (~38,000) and the non-glycosylated product that was obtained *in vitro* (30,000). To this end, a highly purified stroma fraction enriched in CAH1 was obtained and analysed using antibodies specific for β(1,2)-xylose and α(1,3)-fucose epitopes[14] (see Supplementary Information, Fig. S2c–f), which are known to be typical for complex-type *N*-glycans of plants. A number of proteins were immunodecorated with

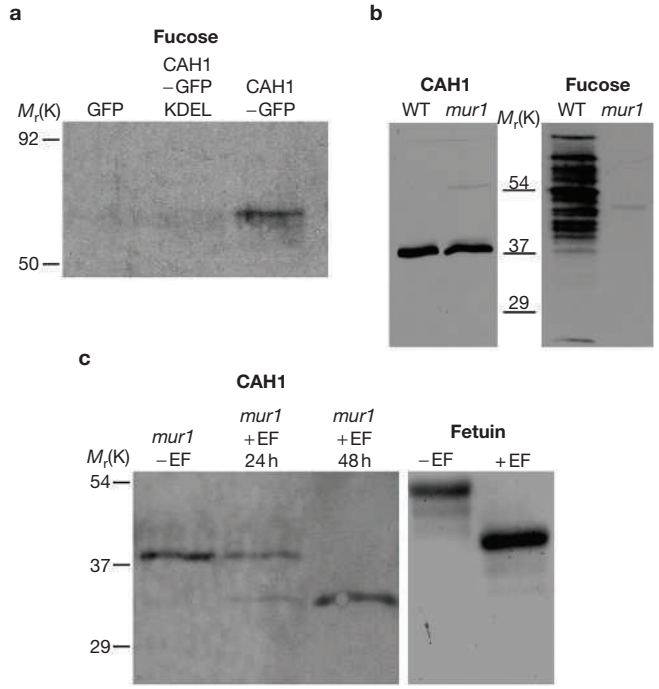

Figure 3 Chloroplast stroma contains an *N*-glycosylated isoform of CAH1. (**a**) Immunoblot with antibodies against α(1–3) fucose residues of CAH1–GFP (green fluorescent protein) fusion protein immunoprecipitated with GFP antibodies from extracts of transformed *Arabidopsis* protoplasts. (**b**) Stroma fractions from wild-type (wt) and mutant *mur1* of *Arabidopsis* were separated by SDS–PAGE electrophoresis and immunoblotted with antibodies against CAH1 (CAH1) and α(1,3)-fucose epitopes (Fucose) at a dilution of 1:1000. (**c**) Deglycosylation of the CAH1 fraction leads to a shift in the electrophoretic mobility of CAH1 protein. A stroma fraction enriched in CAH1 polypeptide was deglycosylated for 24 or 48 h using PNGase F (EF). Fetuin (Sigma) was used as a control for the deglycosylation reaction.

the antibodies, indicating that several stroma proteins are *N*-glycosylated. Prominent spots recognized by the β(1,2)-xylose and α(1,3)-fucose antibodies co-migrated with the spots detected by the CAH1 antibodies (see Supplementary Information, Fig. S2c–f), indicating that the mature stromal CAH1 protein is *N*-glycosylated. We further confirmed *in planta* glycosylation of CAH1 by using the deglycosylating enzyme PNGase F. To circumvent the complication that this enzyme does not work on complex *N*-glycans harbouring α(1,3)-fucose on the proximal glucosamine residue, the experiment was carried out on a stroma fraction that was isolated from the *Arabidopsis mur1* mutant that lacked fucose residues[15,16]. Immunoblots of both wild-type and *mur1* stroma fractions were probed with CAH1 antibodies, revealing a specific CAH1 band of the expected molecular mass in both cases (Fig. 3b, left panel). To establish that the *mur1* mutant provides a suitable tool for PNGase F deglycosylation experiments, and to confirm the specificity of the α(1,3)-fucose antibodies, immunodetection with α(1,3)-fucose antibodies was performed on wild-type and *mur1* mutant stroma fractions (Fig. 3b, right panel). The antibodies crossreacted with several polypeptides in the wild-type stroma fraction, whereas no signal in the expected molecular mass range of CAH1 was observed in the *mur1* stroma fraction (Fig. 3b). After PNGase F treatment, the protein that was recognized by the anti-CAH1 antibody was completely converted into a protein exhibiting an electrophoretic mobility of the non-glycosylated polypeptide (with a relative molecular mass of ~30,000) (Fig. 3c). From this, we concluded that stromal CAH1 protein does indeed harbour complex *N*-glycans.

β(1,2)-xylose and α(1,3)-fucose epitopes are known to be specifically added within the Golgi apparatus[13], implying that transport to and from the Golgi apparatus may represent an intermediate step in CAH1 trafficking to the chloroplast. Therefore, we analysed the effect of brefeldin A (BFA), a fungal antibiotic that inhibits Golgi-mediated vesicular traffic[17], on the intracellular distribution of CAH1–GFP in transiently transformed *Arabidopsis* protoplasts (Fig. 4a–e). Twenty-four hours after transformation, when a fraction of the CAH1–GFP fusion protein had reached the chloroplast (Fig. 4a), BFA (180 μM) was added for 4 h. One hour prior to BFA wash-out, cycloheximide was added to block *de novo* protein biosynthesis. We observed accumulation of CAH1–GFP in the ER and Golgi-like structures following BFA treatment (Fig. 4b, c), which was similar to previous observations made following BFA treatment in *Arabidopsis*[18,19]. Importantly, the CAH1–GFP fusion protein redistributed to the chloroplast after BFA removal. After 3 h, most of the GFP fluorescence colocalized with chlorophyll autofluorescence (Fig. 4d) and, after 4 h, an almost complete colocalization was observed (Fig. 4e). By contrast, a GFP fusion protein that was targeted to the chloroplast via the Toc/Tic system was not affected by BFA treatment (see Supplementary Information, Fig. S3a, b).

We also followed the fate of endogenous CAH1 protein following BFA treatment by using subcellular fractionation studies (Fig. 4f). *Arabidopsis* suspension cells were analysed after 4 h in the absence (Fig. 4f, lanes 1, 2, 6 and 7) and presence (Fig. 4f, lanes 3, 4, 5, 8, 9 and 10) of BFA. In the absence of BFA, the mature CAH1 form accumulated in the soluble fraction that contained all the stroma content (Fig. 4f, lane 1). Under these conditions, a low-molecular-mass form, presumably corresponding to the unglycosylated CAH1 precursor, was found in the microsomal fraction (Fig. 4f, lane 6). Following BFA treatment (Fig. 4f, lanes 3 and 8), presence of the mature CAH1 form in the soluble fraction was reduced (Fig. 4f, lane 3) and a concomitant accumulation of partially glycosylated CAH1 forms was observed in the microsomal fraction (Fig. 4f, lane 8). Separation of this fraction from both control and BFA-treated cells by sucrose density gradients showed that these CAH1 forms were localized in light dense microsomes, particularly in ER-enriched fractions (data not shown). As for the CAH1–GFP localization analysis, cycloheximide was added to the cell culture after 3 h of BFA incubation. After an additional hour of incubation with both drugs, BFA was washed out using a solution that still contained cycloheximide. Three hours after BFA wash-out, the high-molecular-mass forms of CAH1 disappeared from the microsomal fraction (Fig. 4f, lane 9), whereas the mature CAH1 form started to accumulate in the soluble fraction that contained the stroma (Fig. 4f, lane 4). At 4 h after BFA removal, the mature form of CAH1 was present in the soluble fraction and absent from the microsomal fraction (Fig. 4f, lanes 5 and 10), a distribution that was similar to that of the time point before BFA treatment (Fig. 4f, lanes 1 and 6). Chloroplast-localized CAH1 was almost completely absent after BFA treatment, which might indicate a rapid turnover of the CAH1 protein under certain conditions. A potential regulation of CAH1 by protein degradation will therefore provide an interesting objective for future studies. Taken together, these results strongly indicate that CAH1 targeting to the chloroplast involves a BFA-sensitive vesicular transport pathway.

In conclusion, our data provide strong evidence that the chloroplast proteome contains *N*-glycosylated proteins that are transported through the ER, in addition to the proteins synthesized in the chloroplast and those transported through the Toc/Tic apparatus. Recently, extensive studies of the chloroplast proteome have revealed the occurrence of many proteins

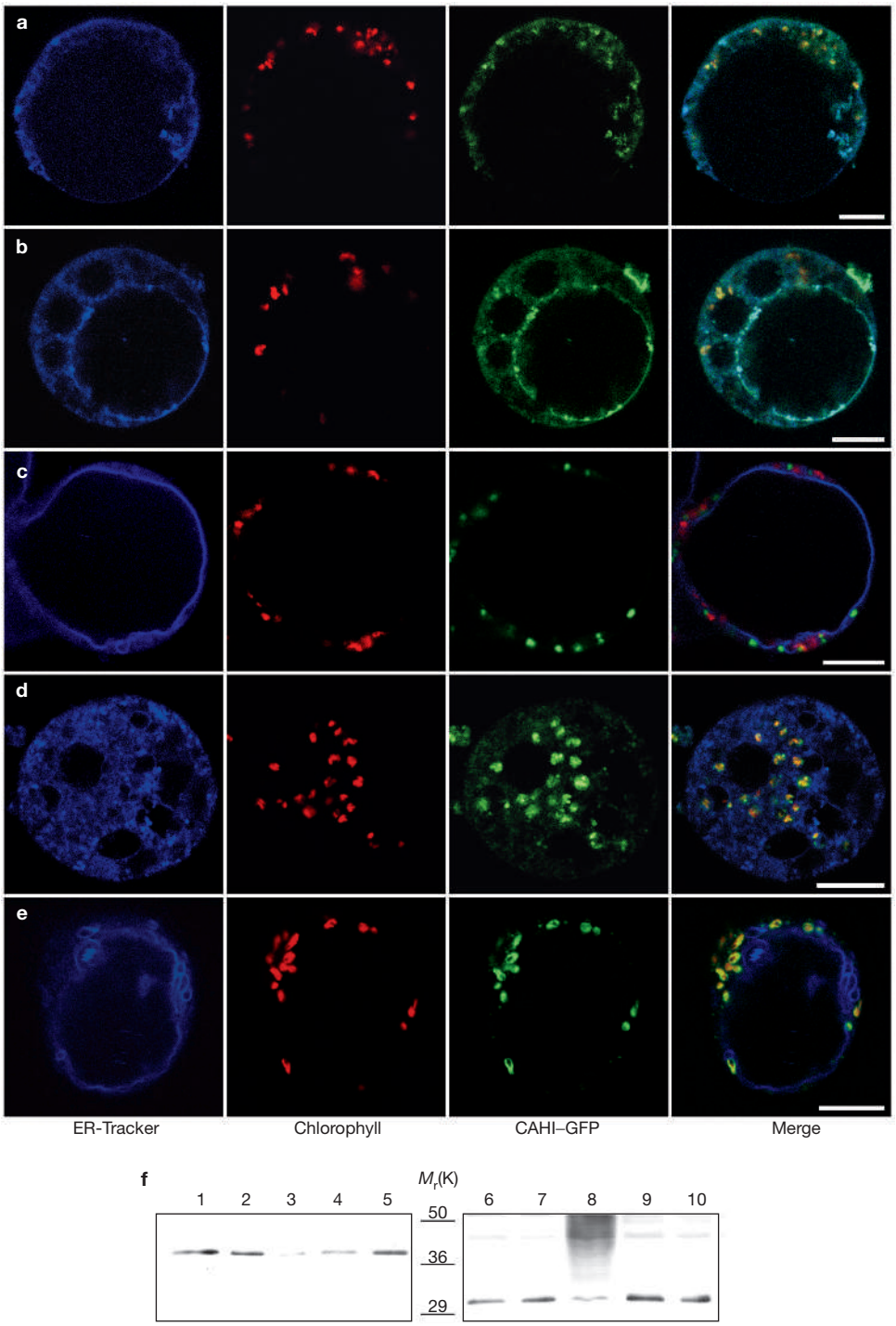

Figure 4 Effect of BFA on chloroplast targeted CAH1–GFP fusion construct in *Arabidopsis* protoplasts (**a–e**) and native CAH1 in *Arabidopsis* cell suspensions (**f**). (**a**) Twenty-four hours after transformation, CAH1–GFP (green fluorescent protein) is mainly localized in the endoplasmic reticulum (ER) but also begins to reach the chloroplast, as revealed by colocalization of the fluorescence with that of the ER-Tracker and chlorophyll. (**b**, **c**) Treatment with 180 μM brefeldin A (BFA) for 4 h causes the redistribution of CAH1–GFP to the ER and Golgi-like structures. (**d**) Three hours after removal of BFA and in the presence of 50 μM cycloheximide, CAH1–GFP fusion was mainly relocated to the chloroplast. (**e**) Four hours after removal of BFA and in the presence of 50 μM cycloheximide, CAH1–GFP fusion was completely located in the chloroplasts. Scale bar, 10 μm. (**f**) Effect of BFA on the distribution of native CAH1 between soluble, stroma-containing (lanes 1–5) and microsome-enriched (lanes 6–10) fractions of *Arabidopsis* suspension cells. *Arabidopsis* cell suspensions were treated for 4 h in the absence (lanes 1, 2, 6 and 7) and presence (lanes 3, 4, 5, 8, 9 and 10) of 180 μM BFA. Control cells were incubated for 5 h without (lanes 1 and 6) and with (lanes 2 and 7) 100 μM cycloheximide. After 4 h of treatment with BFA, CAH1 disappeared from the supernatant, whereas different isoforms accumulated in the microsome fraction (lanes 3 and 8). At this time, BFA was removed and cells were grown in cycloheximide-containing media (added 1 h before removal of BFA) for 3 h (lanes 4 and 9) and 4 h (lanes 5 and 10). The mature CAH1 form is completely relocated in the stroma fraction 4 h after removal of BFA. All the samples were immunoblotted with antibodies against CAH1. Antimycine-A-resistant NADH cytochrome *c* reductase activity, a marker for ER, was measured in both fractions (see Supplementary Information, Fig. S3c).

with predicted SP inside chloroplasts[6,7]. The results presented here provide firm functional support for the existence of such a novel protein-targeting pathway through the secretory system to the chloroplast in plants.

When the first genes were transferred from the genome of the cyanobacterial endosymbiont to the nuclear genome, there was probably no protein-sorting system for the ancestral chloroplast. The encoded proteins may have been secreted from the eukaryotic host and subsequently taken up by the endosymbiont. Many algal groups, as well as apicomplexan parasites[20,21], possess a so-called 'complex' plastid that has originated from a secondary endosymbiotic event[22]. The accepted mechanism whereby proteins are routed to these complex plastids is through the secretory pathway[20].

As shown here, this ancestral pathway seems to have been maintained for some chloroplast proteins. The mechanism whereby these plastid proteins are translocated from the secretory pathway to the chloroplast is intriguing. In apicomplexan parasites and diatoms, plastid proteins that are targeted through the secretory pathway contain a bipartite amino-terminal pre-sequence that consists of a SP followed by a transit peptide[23,24]. By contrast, *Arabidopsis* CAH1 is only predicted to contain an N-terminal SP, indicating that the translocation mechanism is not identical. However, the C terminus of CAH1 is highly hydrophilic and enriched in lysine residues, such as the apicomplexan transit peptides[23]. Therefore, the C terminus could possibly serve as a signal sequence in this particular uptake mechanism.

Although our results strongly indicate that vesicular transport through the secretory system provides the mechanism by which CAH1 is transported into the plastid, it remains to be determined how precisely this is accomplished. For example, evidence for direct contact between the ER and the chloroplast membranes has been previously obtained[25] and biochemical interactions between the two membrane systems are essential for lipid metabolism[26]. Future detailed studies will be needed to resolve the exact mechanism that is used for transporting CAH1 to the chloroplast. These may further reveal whether the pathway outlined here also accommodates chloroplast localization of other proteins, such as rice α-amylases[8,27]. Based on indirect evidence, the authors hypothesized that vesicular transport through the Golgi apparatus is involved in the plastid targeting of these α-amylases. However, the susceptibility of the plastid isoform of α-amylase I-1 to deglycosidases[8] indicates that this protein does not traffick through the Golgi. Therefore, dual import of rice α-amylases to plastids and extracellular space could occur via two mutually exclusive conserved targeting mechanisms, as has been shown for other proteins[28]. In contrast to these previously reported findings, CAH1 is mainly localized in the chloroplast and is not subjected to dual targeting. Taken together, our data convincingly demonstrate transport of a protein to the chloroplast through the secretory system in plants.

METHODS

Plant material and growth conditions. Wild-type *Arabidopsis thaliana*, ecotype Columbia, and the mutant *mur1* plants were grown in a growth chamber set at 23/18 °C day/night temperatures, 70% humidity and a photon flux density of 150 μmol m^{-2} s^{-1} during the 8-h photoperiod.

Cloning. A putative α-CA EST clone (*Arabidopsis thaliana*, GenBank accession number Z18493) was used to screen a total of 3.0×10^5 plaques from a Uni-ZAP™ XR *Arabidopsis thaliana* cDNA library (Stratagene, La Jolla, CA). Nucleotide sequences of three positive clones were determined and the 5′ end of the cDNA was identified through the 5′-RACE–PCR procedure (Gibco-BRL, Carlsbad, CA). A genomic library was also screened and three positive clones were subcloned. A fragment including the 5′-end of the gene and 728 base pairs upstream of the putative translation initiation site was sequenced.

Overexpression of recombinant CAH1 in *Escherichia coli*. Polymerase chain reaction (PCR) was used to amplify a selected cDNA region from *CAH1* and cloned into *BamH*I–*Xho*I-digested expression vector pET23a (+) (Novagen, Madison, WI). The resulting plasmid, pSLaCAH1, was verified by sequencing and encodes a recombinant *Arabidopsis* CAH1 starting at Gly 28, with an N-terminal T7-tag and a C-terminal 6-histidine tag. The construct was transformed into *E. coli* BL21 (DE3) and the expressed recombinant protein purified under denaturing conditions to near-homogeneity, using a histidine tag-binding resin, according to the pET System Manual (Novagen).

Chloroplast isolation and fractionation. Chloroplasts from the *A. thaliana* wild-type and the *mur1* mutant were purified as described previously[29]. The chloroplasts were further purified on a 50% (v/v) Percoll gradient (Pharmacia Biotech, Uppsala, Sweden). Intact chloroplasts in chloroplast resuspension buffer were sonicated for 3×30 s and centrifuged at 15,000 *g* for 30 min. The supernatant, mainly containing stroma proteins, was applied to a 1 ml MonoQ anion exchange column (HiTrap Q FF; Pharmacia Biotech) that was equilibrated with 20 mM Tris-HCl buffer (pH 7.8). Bound proteins were eluted with a 30 ml linear gradient from 0 to 800 mM NaCl. Each fraction was desalted using PD-10 columns (Pharmacia Biotech). The purification process was monitored by subjecting aliquots from each fraction to western blot analyses.

Immunocytochemistry. Developing *Arabidopsis* leaves were cut into 2 mm^2 pieces in freshly made 4% paraformaldehyde and 0.5% glutaraldehyde in a sodium phosphate sucrose buffer (0.1 M phosphate buffer, 0.05 M sucrose, pH 7.2) and fixed for 5 h at room temperature under a gentle vacuum. After several rinses, samples were dehydrated through a graded ethanol series and embedded in LR white resin (London Resin Co Ltd, London, UK). Immunolocalization at the light microscope level was carried out on 1–2 mm tissue sections, cut with a diamond knife on an LKB superfrost-plus microtome and then affixed to slides. CAH1-specific antibodies at a dilution of 1:100 were used and the primary immune complexes were visualized by probing the sections for 2 h with colloidal-gold-conjugated (6 nm) goat anti-rabbit immunoglobulin G (IgG; diluted 1:100). The immuno-label was enhanced by using a silver enhancement kit (Biocell, Cardiff, UK). Sections were then counter-stained with toluidine blue and permanently mounted for observation on a Zeiss Axiophot microscope using bright-field illumination. Immunolocalization at the electron-microscopy level was carried out on 150 nm ultra-thin sections that were picked up on uncoated 200-mesh nickel grids. The gold labelling was examined on an electron microscope after staining the grids in 2% aqueous uranyl acetate for 10 min.

Expression in reticulocyte lysate in the presence of dog pancreas microsomes. The *CAH1* gene and the N-terminally truncated version (lacking positions 1–-24) were cloned into pGEM1 (Promega, Madison, WI) with the initiator ATG codon in the context of a 'Kozak consensus' sequence[30]. The constructs were transcribed by SP6 RNA polymerase (Promega) for 1 h at 37 °C. The transcription mixture was as follows: 1–5 μg DNA template, 5 μl 10 × SP6 H-buffer (400 mM Hepes-KOH (pH 7.4), 60 mM Mg acetate, 20 mM spermidine-HCl), 5 μl bovine serum albumin (1 mg ml^{-1}), 5 μl m7G (5′) ppp (5′) G (10 mM) (Pharmacia), 5 μl DTT (50 mM), 5 μl rNTP mix (10 mM ATP, 10 mM CTP, 10 mM UTP, 5 mM GTP), 18.5 μl H_2O, 1.5 μl RNase inhibitor (50 units), 0.5 μl SP6 RNA polymerase (20 units). Translation was performed in reticulocyte lysate in the presence or absence of dog pancreas microsomes[31]. The acceptor peptide Benzoyl-NLT-methylamide (Quality Control Biochemicals Inc., Hopkinton, MA) was added as a competitive inhibitor of glycosylation at a final concentration of 200 μM. For protease treatment, 5 μl 4.5 mg ml^{-1} proteinase K and 1 μl 200 mM $CaCl_2$ was added to the translation mix and the sample was incubated on ice for 30 min. The treatment was stopped by addition of 0.5 ml 20 mg $^{-1}$ml PMSF. Translation products were analysed by SDS–PAGE and gels were quantified on a Fuji FLA-3000 phosphoimager using Fuji Image Reader 8.1j software.

Construction of GFP reporter plasmids for transient expression in *Arabidopsis* cells. The GFP reporter plasmid, CaMV35S-sGFP(S65T), and the plasmid containing the transit peptide (TP) sequence from RBCS fused to GFP, (CaMV35S-TP-sGFP(S65T)), have been described previously[32]. DsRed2-ER protein (BD

Biosciences ClonTech, Palo Alto, CA) was used as an ER control. The plasmids for expression of the full or truncated *Arabidopsis* CAH1 protein that were fused to GFP were constructed as follows: CaMV35S–CAH1–sGFP(S65T) corresponding to the coding region of *Arabidopsis* CAH1 was PCR amplified using the two flanking primers for *Sal*I (TAAAAGTCGACATGAAGATTATGATGATGA) and rev1-*Nco*I (AAAACCCATGGAATTGGGTTTTTTCTTTTT), and the PCR product was cloned into the *Sal*I-*Nco*I-digested GFP reporter plasmid CaMV35S-sGFP(S65T). The protocol was similar for the other constructs. CaMV35S–(1–40)CAH1–sGFP(S65T) corresponding to CAH1 containing the first 40 amino acids was PCR amplified using the two flanking primers for *Sal*I and rev2-*Nco*I (GTGTCCCATGGGGTTTGGTCCATTTTTGCC). The CaMV35S–(1–40)CAH1–sGFP(S65T)–KDEL corresponding to CAH1 containing the first 40 amino acids fused to a KDEL-tagged GFP was PCR amplified using the two flanking primers for *Sal*I and rev1-*BsrG*I (TCTGCTGTACAGTCAGAGTTCATCCTTATACAGCTCGTCCATGCC). The CaMV35S–CAH1–sGFP(S65T)–KDEL corresponding to the coding region of *Arabidopsis* CAH1 fused to a KDEL-tagged GFP and was PCR amplified using the two flanking primers for *Sal*I and rev1-*BsrG*I. The plasmids were sequenced to ascertain that the orientation and sequences of the inserted fragments were correct. The plasmids used for PEG transformation were prepared using the Plasmid MidiPrep kit (Bio-Rad Laboratories, Hercules, CA).

Transformation and confocal laser scanning microscopy. Five micrograms of the appropriate plasmid constructs were introduced into *Arabidopsis* cells using the PEG method. After transformation, cells were incubated on the plates for 24–48 h in the dark. Cells were transferred to glass slides and examined by fluorescence microscopy. Localization of GFP, GFP fusions and DsRed2-ER was assessed in transformed cells by confocal laser scanning microscopy, as outlined in the Supplementary Information.

ER-Tracker fluorescence analysis. *Arabidopsis* cells expressing GFP and non-transformed cells were centrifuged and resuspended in a medium containing 1 μM ER-Tracker blue-white DPX (Molecular Probes, Eugene, OR`). After 45 min incubation in this medium at room temperature, cell suspensions were centrifuged and resuspended in fresh medium without dye. Cells were transferred to glass slides and observed by confocal laser scanning microscopy, as outlined in the Supplementary Information.

Separation of intracellular membranes by density gradient centrifugation. Isolation of the total microsome fraction and separation by density gradient centrifugation was carried out as described previously[33]. Briefly, 10 g of packed *Arabidopsis* cells frozen in liquid nitrogen were ground in a mortar and pestle, resupended in two volumes of homogenization buffer (25 mM Tris-HCl, pH 7.5, 0.25 M sucrose, 3 mM EDTA, 1 mM DTT) and centrifuged for 15 min at 10,000 *g* at 4 °C. The supernatant was centrifuged for 60 min at 150,000 *g*, the supernatant (SN) collected, and the pellet (termed total microsomes) was thoroughly resuspended in 1 ml of buffer containing 5 mM Tris-HCl, pH 7.5, 0.25 mM sucrose, 3 mM EDTA and 1 mM DTT, and loaded into an 11 ml linear gradient of 20–50% (w/w) sucrose buffered with 5 mM Tris-HCl, pH 7.5, 3 mM EDTA and 1 mM DTT. Sucrose gradients were centrifuged at 80,000 *g* for 5 h at 4 °C in a swing-out rotor (SW41 Beckman). Fractions (1 ml) were collected and stored at –80 °C until analysis.

Deglycosylation assays. A stroma fraction (100 μg protein/ml) enriched in CAH1 protein isolated from the mutant *mur1* of *Arabidopsis thaliana* was deglycosylated using a recombinant peptide-*N*-glycosidase F (PNGase F, Roche Diagnostics Corporation, Mannheim, Germany), according to the manufacturer's instructions with some modifications. The sample was denatured at 100 °C for 5 min in the presence of 1% (w/v) SDS. After cooling the sample to room temperature, SDS was removed using the SDS-out kit (Pierce Co., Rockford, IL). The sample was then diluted with the same volume of 0.1 M Tris-HCl buffer (pH 7.8) containing 0.5% (v/v) Nonidet P-40 (Sigma, St Louis, MO). Twenty units of PNGase F were added and the sample was incubated for 24 or 48 h at 37 °C. The sample was further analysed by SDS–PAGE and immunoblotting with antibodies against CAH1. Fetuin (Sigma) was used as a positive control during the deglycosylation experiments and treated identically to the stroma fraction.

Immunoprecipitation reactions. Transfected *Arabidopsis* protoplasts were resuspended in extraction buffer containing 25 mM Tris-HCl (pH 7.8), 10 mM $MgCl_2$, 5 mM EGTA, 2 mM DTT, 10% glycerol, 75 mM NaCl, 60 mM β-glycerophosphate, 0.2% Nonidet P-40, 1 mM benzamidine and 1× Protease Inhibitor Cocktail (Sigma). The resuspended protoplasts were frozen in liquid nitrogen and then thawed and clarified by centrifugation. Extracts were first precleared for 2 h at 4 °C with normal mouse IgG Protein G Sepharose or normal rabbit IgG Protein A Sepharose beads for anti-GFP (mouse monoclonal antibody sc-9996; Santa Cruz Biotechnology) and anti-CAH1 polyclonal antibodies, respectively. CAH1 fusion proteins were next precipitated by incubating the precleared extracts either with 1.5 μg of anti-GFP or anti-CAH1 antibodies for 4 h at 4 °C and then capturing the immunocomplexes on 10 μl of Protein G or Protein A Sepharose beads. Beads were washed with buffer (50 mM Tris-HCl, 150 mM NaCl, 0.1% NP 40, pH 7.5) and bound proteins were eluted by boiling in 25 μl Laemmli sample buffer.

Brefeldin-A treatment and recovery of transformed protoplasts and cell suspensions. Stock solutions of BFA (Sigma) were prepared at 36 mM by dissolving BFA in DMSO. Aliquots of this stock were added to 3- to 4-day-old cell suspensions and/or to 24-h-old protoplast suspensions to give a final concentration of 180 μM. Cells were incubated with BFA for 4 h under continuous agitation. BFA-treated cells or protoplasts were harvested by low-speed centrifugation. For recovery experiments, *Arabidopsis* cells or transformed protoplasts were treated with 180 μM BFA for 4 h. Forty-five minutes prior to the end of the incubation period, cycloheximide was added to a final concentration of 50 and 100 μM for protoplasts or cells, respectively. Cells and protoplasts were washed twice with BFA-free, cycloheximide-containing culture medium, resuspended in the same medium, and samples were harvested at different times during recovery.

Note: Supplementary Information is available on the Nature Cell Biology website.

ACKNOWLEDGEMENTS

We thank A. Kraut for technical assistance in the transient expression of GFP fusions in plant cells; L. Faye for the gift of antibodies against xylose and fucose residues; C. Robinson for helpful discussion; and J. Brangeon and R. Boyer for technical help in the immunocytochemistry experiments. The authors are grateful to B. Martin for critical reading of the manuscript. This work was supported by grants from the Swedish National Research Council, FORMAS, Wallenberg and Kempe Foundations, and the Swedish Foundation for Strategic Research.

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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ERRATUM

Owing to a technical error, the pages of this manuscript were originally mis-numbered by a 100 pages. This has now been corrected online. The corrected online manuscript is numbered 100 pages higher than the mis-numbered version.

Evidence for a protein transported through the secretory pathway en route to the higher plant chloroplast

Supporting Online Material

Supplementary Methods and Results

In vitro import assays. All precursors (the small subunit of pea ribulose bisphosphate carboxylase/oxygenase (SSU), and both the full-length CAH1 and a truncated version lacking the signal peptide) were generated with a rabbit reticulocyte TNT-coupled transcription and translation system (Promega, Madison, WI, USA) supplied with ^{35}S-methionine. Chloroplast import reactions were carried out as previously described[1,2].

Analysis of glycosylated proteins in the chloroplast stroma of *Arabidopsis*. The stroma from *Arabidopsis* chloroplasts was fractionated by anion exchange chromatography. Stroma samples containing 300-400 g of protein were precipitated with 0.15 % (v/v) deoxycholic acid and 72 % (v/v) TCA as previously described[3] and solubilized in 2D rehydration solution, containing 8 M urea, 2 % (w/v) CHAPS, and 0.002 % (w/v) bromophenol blue. The solubilized samples were loaded onto linear immobilized pH gradient gels (IPG) covering the pH ranges from 4-7 and 3-10 (Amersham Pharmacia Biotech AB, Uppsala, Sweden). The samples were applied by in-gel-rehydration and isoelectrically focused using an IPGphor system (Amersham Pharmacia Biotech AB). After focusing, the strips were equilibrated twice for 15 min in equilibration buffer (50 mM Tris-HCl (pH 8.8), 6 M urea, 30 % (v/v) glycerol, 0.002 % (v/v) bromophenol blue, and 2 % (w/v) SDS), containing 1 % (w/v) DTT in the first equilibration and 2.5 % (w/v) iodoacetamide in the second. After the equilibration steps, the strips were loaded onto 10 % SDS-PAGE gels, and

electrophoretically separated at constant current. After 2D protein separation, stroma proteins were detected using a silver-staining method as described before[4], or they were electrotransferred onto nitrocellulose membranes. The membranes were then incubated with antibodies raised against CAH1, β(1,2)-xylose, and α(1,3)-fucose epitopes.

Southern and northern blot analysis. Genomic DNA was extracted from developing *Arabidopsis* leaves, according to the method of Moore[5] and total RNA was isolated from developing *Arabidopsis* leaves and roots according to the protocol of Verwoerd *et al*[6]. Northern blot analysis was performed as previously described[7].

Antibody production. Polyclonal antibodies were raised against recombinant *Arabidopsis* CAH1 (Agri Sera AB, Vännäs, Sweden). The antibodies were purified using CAH1-coupled Affigel-10 (Bio-Rad, Hercules, CA,USA), following the manufacturer's recommendations.

Determination of chlorophyll and enzymatic markers. Chlorophyll concentrations were determined in 80 % acetone according to the method of Porra *et al.*[8]. Thermolysin treatments of intact chloroplasts were performed on ice for 30 min in 40 µl reaction volumes (10 µg chlorophyll in chloroplast resuspension buffer), using 200 µg/ml thermolysin (Boehringer Mannheim). NADH cytochrome *c* reductase (± Antimycine A) activity was determined as described by Hodges and Leonard[9].

Western blot analysis. Crude protein extracts were prepared from *Arabidopsis* leaves and roots as previously described[10]. Protein concentration was determined using the Bio-Rad Protein Assay (Bio-Rad). SDS-PAGE was conducted following the protocol of Laemmli[11].

Confocal laser scanning microscopy. Fluorescent proteins, ER-Tracker Blue-White DPX (Molecular Probes/Invitrogen, Eugene, OR,USA?) and chlorophyll fluorescence were detected employing a Leica TCS SP2 AOBS confocal laser scanning system mounted on a Leica DM IRE2 inverted microscope (Leica Microsystems, Mannheim, Germany). ER tracker was excited at 364 nm with an Argon UV laser. Fluorescence was detected at 410- 475 nm by the in between line sequential scan mode to separate it from GFP fluorescence excited at 488 nm with an Argon laser

and detected between 490-550 nm and as chlorophyll fluorescence detected between 650-740 nm. DsRed2-ER was excited at 561 nm with a diode pumped solid state laser and fluorescence detected between 563-628 nm by in between sequential line scanning. Detection was at maximum system resolution at 185 nm in xy and 390 nm in the z direction employing an HCX PLAPO CS 63x oil objective NA 1.4 and a pixel sampling size from 70-90 nm.

Supplementary Results

Purity of chloroplast preparations

Chloroplasts from *Arabidopsis thaliana* were purified as previously described[12]. High purity of these chloroplasts was confirmed by proteomic analysis, showing that less than 5% of the identified *Arabidopsis* proteins corresponded to non-plastid proteins[12].

The specific activity of the peroxisomal marker hydroxipyruvate reductase (EC 1.1.1.81) was barely detectable in chloroplasts suspensions (6.8 nmol min^{-1} mg $protein^{-1}$) compared to the specific activity measured in crude leaf extracts (256 nmol min^{-1} mg $protein^{-1}$). The specific activity of the mitochondrial marker fumarase (EC 4.2.1.2) was undetectable in our chloroplast preparations, indicating that contamination by these organelles was insignificant. The same results were obtained when the specific activity of the ER marker antimycine A-resistant NADH-cytochrome *c* reductase was measured. All these results, together with the data from the proteomic analysis[12], indicate high purity of our chloroplast preparation. In addition, immunoblotting analysis of the different sub-plastid fractions with antibodies raised against markers of these fractions showed that there was no cross-contamination (Fig. S1b).

Other *N*-glycosylated proteins are present in the stroma

Antibodies raised against CAH1 cross-reacted with a protein at ~38 kDa with a variable pI value ranging from 5.2 to 5.6 (Fig. S2d). Antibodies raised against β(1,2)-xylose and α(1,3)-fucose cross-reacted with the same protein recognized by the CAH1 antibodies (Fig. S2e and f), suggesting that the mature stromal CAH1 protein is *N*-glycosylated.

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Figure S1. CAH1 is localized in the *Arabidopsis* chloroplast stroma. (**a**) Immunogold labelling of CAH1 in *Arabidopsis* leaves. Sections were examined under the light microscope. Labelling was carried out with a pre-serum. The scale bar is 20 m long. (**b**) Immunoblotting of total cell extracts (CE), intact chloroplasts (C), envelope membranes (E), stroma (S), and thylakoid membranes (T), with antibodies (dilution 1:1000) against CAH1, light-harvesting-complex protein (LHCP), and the envelope ceQORH protein (ceQORH). Twenty micrograms of protein were loaded in each lane. (**c**) Immunoblotting with antibodies against CAH1 protein of intact chloroplasts (lane 1), intact chloroplasts treated with thermolysin (lane 2), and broken chloroplasts treated with thermolysin (lane 3). Three micrograms of protein were loaded in each lane. (**d**) CAH1 is taken up into the ER. The full-length CAH1 was fused to the N-terminus of a KDEL-tagged GFP. As a subcellular localization control, cells were co-transformed with a plasmid containing the DsRed2-ER protein (BD Biosciences ClonTech, Palo Alto, CA, USA), scale bar 10 m. (**e**) The full-length CAH1 and a truncated version lacking the ER signal peptide are not taken up into chloroplasts. In vitro uptake experiments into pea chloroplasts is shown for SSU (lanes 1-3), the full-length CAH1 (lanes 4-6), and a truncated version of CAH1 lacking the ER signal peptide (lane 7-9). The precursors were incubated without (-) or with (+) pea chloroplasts (Chl) either with no additions (lanes 2, 5, and 8) or with addition of thermolysin (T) (lanes 3, 6, and 9). The precursor and the processed form of each protein are indicated by white and black dots, respectively.

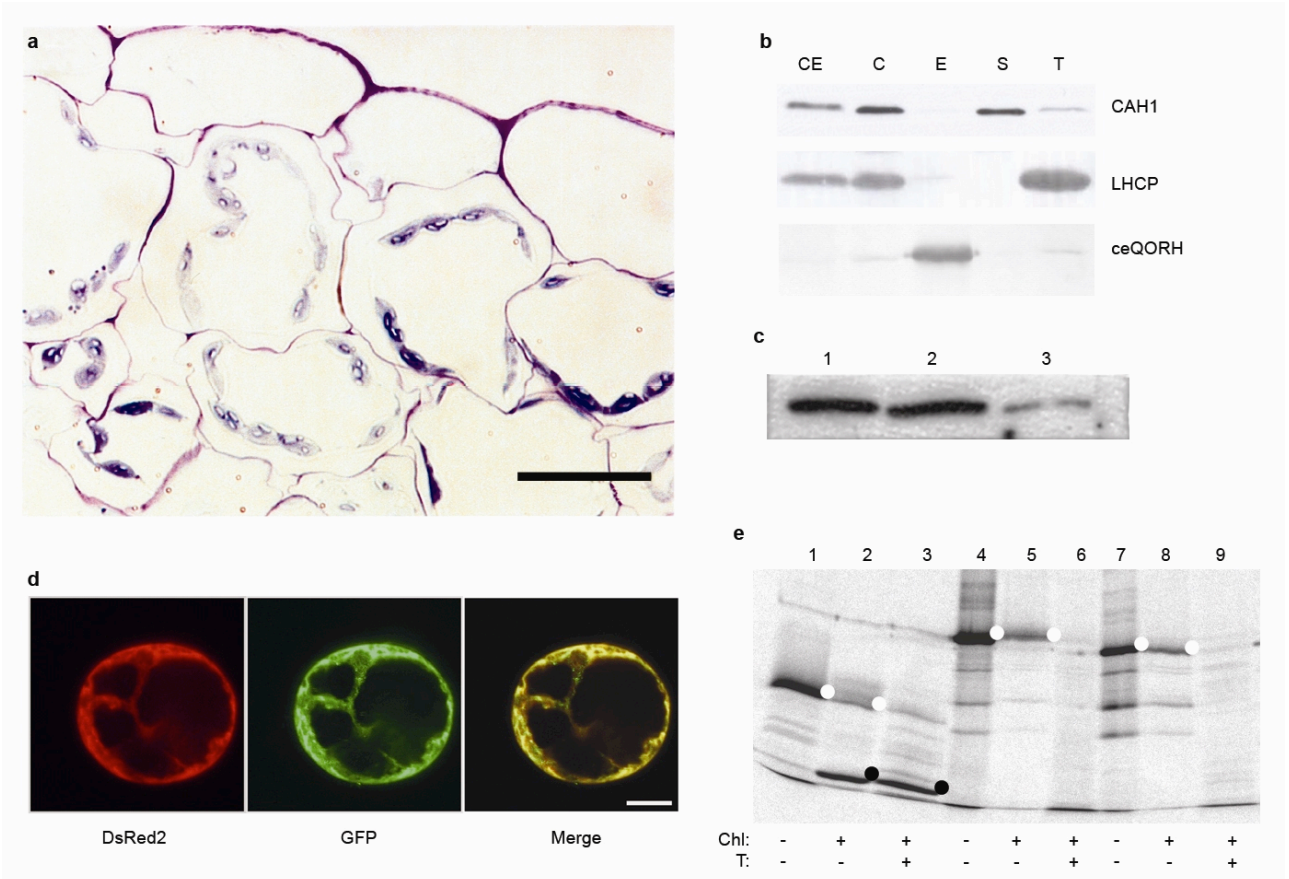


Figure S2. Immunoblot analysis of protein extracts with antibodies raised against CAH1 **(a)** and GFP **(b).** The proteins were extracted from protoplasts transformed with GFP, CAH1-GFP-KDEL, and CAH1-GFP and immunoprecipitated with antibodies raised against GFP **(a)** and CAH1 **(b).** The stroma CAH1 isoform is N-glycosylated. (**c, d, e, and f**) CAH1 from isolated chloroplast stroma was purified by anion exchange chromatography and eluted with 0.25 M NaCl. This fraction was further fractionated by 2D-gel electrophoresis (pH range 4-7; 75 g protein), silver stained (**c**) or immunoblotted with antibodies against CAH1 (**d**), β(1,2)-xylose (**e**), and α(1,3)-fucose N-glycan residues (**f**). All antibodies cross-reacted with the same protein, indicating that mature stroma CAH1 is glycosylated. Areas containing proteins that cross-reacted with all three antibodies are circled.

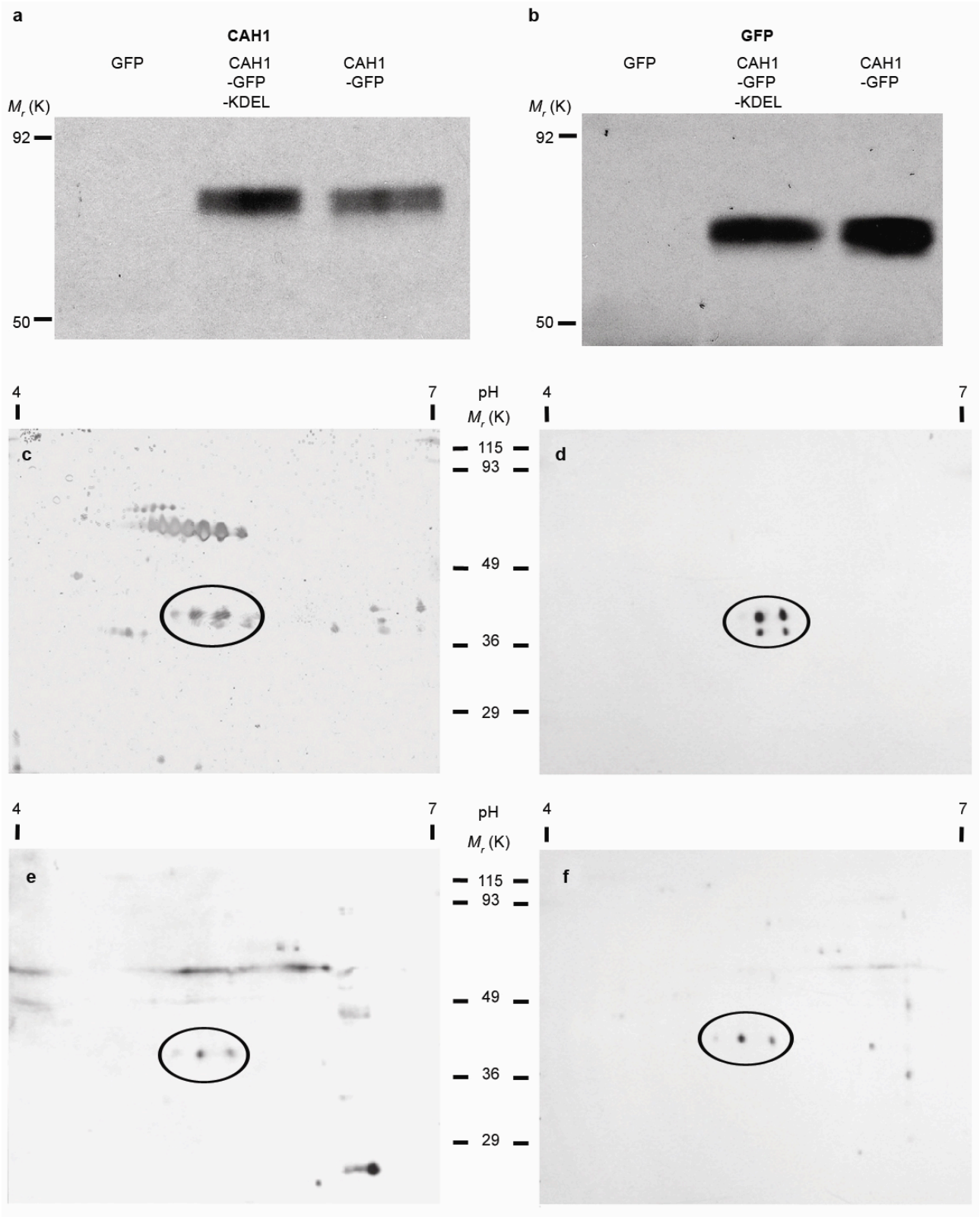
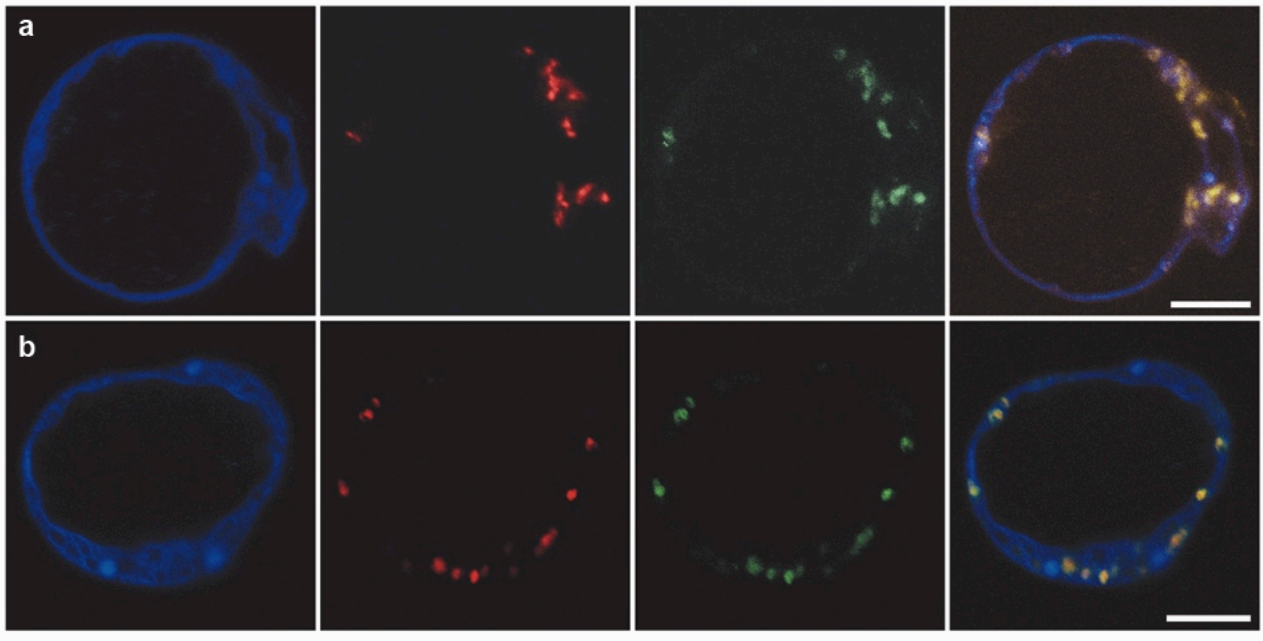


Figure S3. Targeting to the chloroplast through the Tic/Toc system is not affected by BFA treatment. *Arabidopsis* protoplasts transformed with TP-GFP were incubated in the presence of 180 M BFA for 4 h (**a**), then BFA was removed and protoplasts were grown in in fresh medium for additional 4 h in the presence of 50 uM cycloheximide (**b**), scale bar 10 m. Antimycine A resistant NADH cytochrome *c* reductase activity, a marker for ER, was measured in both the supernatant and the microsome fraction from the different treatments described in Fig. 5f. Activity was expressed as nmol NADH reduced mg^{-1} min^{-1}(**c**).



c

Treatment	Supernatant	Microsomal fraction
Control	16 ± 1	160 ± 10
Control + CHX	18 ± 0.5	158 ± 8
BFA	25 ± 2	155 ± 12
After BFA + CHX, 3h	21 ± 1	150 ± 9
After BFA + CHX, 5h	21 ± 1	152 ± 5
Alter BFA without CHX, 5h	12 ± 0.5	148 ± 10