A considerably small fraction of approximately 60–100 proteins of all chloroplast proteins are encoded by the plastid genome. Many of these proteins are major subunits of complexes with central functions within plastids. In comparison with other subcellular compartments and bacteria, many steps of chloroplast protein biogenesis are not well understood. We report here on the first study of chloroplast-localised trigger factor. In bacteria, this molecular chaperone is known to associate with translating ribosomes to facilitate the folding of newly synthesized proteins. Chloroplast trigger factors of the unicellular green algae *Chlamydomonas reinhardtii* and the vascular land plant *Arabidopsis thaliana* were characterized by biophysical and structural methods and compared to the *Escherichia coli* isoform. We show that chloroplast trigger factor is mainly monomeric and displays only moderate stability against thermal unfolding even under mild heat-stress conditions. The global shape and conformation of these proteins were determined in solution by small-angle X-ray scattering and subsequent *ab initio* modelling. As observed for bacteria, plastidic trigger factors have a dragon-like structure, albeit with slightly altered domain arrangement and flexibility. This structural conservation despite low amino acid sequence homology illustrates a remarkable evolutionary robustness of chaperone conformations across various kingdoms of life.

The transformation of the one-dimensional genetic information into complex protein structures is a challenging task for cells. Polypeptides that emerge vectorially from translating ribosomes cannot fold completely to the native conformation unless the polypeptide chain is fully synthesized and released from the ribosome. During this process, nascent polypeptides are highly susceptible to premature folding, misfolding, and aggregation. In addition, polypeptides synthesized on cytosolic ribosomes and destined for organelles need to be recruited early and kept in an unfolded state to facilitate their translocation across subcellular membranes. Thus, cells have acquired various factors associating with translating ribosomes to assist protein maturation, to prevent premature folding, and to mediate membrane targeting for translocation. Many of these factors belong to the abundant and structurally diverse family of molecular chaperones, which act at all stages of a protein’s life span to promote a balanced protein homeostasis. Across all kingdoms of life, specific molecular chaperones associate with translating ribosomes to guide the initial steps of de novo folding of nascent polypeptides. Further downstream, other chaperones take over nascent polypeptides to assist folding and final maturation. In bacteria, the ATP-independent trigger factor (TF) is the predominant chaperone that transiently binds to ribosomes. This association is mediated by the ribosomal protein Rpl23, which positions TF next to the ribosomal exit tunnel for early binding of nascent polypeptides. A comprehensive analysis of TF substrates indicated that the chaperone binds most newly translated polypeptides. In fact, *E. coli* cells contain a two- to three-fold molar excess of TF relative to ribosomes, thus providing sufficient chaperones to act on all translating ribosomes. High-resolution structural analyses of various bacterial TF molecules showed that TF adopts a unique elongated conformation resembling a ‘crouching dragon’ with three domains. The N-terminal domain is responsible for ribosome association and contains an essential signature motif for this interaction. This domain further supports
the C-terminus to shape the backbone structure of TF. The C-terminal domain is the main module of TF and possesses in vitro chaperone activity on its own. This domain folds back to interact with the N-terminus and forms the backbone of TF with an open cavity and two protruding arms. Within the amino acid sequence, both termini are separated by the peptidyl–prolyl cis–trans isomerase (PPIase) or so-called head domain, which is situated opposite of the N-terminus in the three-dimensional structure. In contrast with other molecular chaperones, which contain one specific substrate binding site, TF seems to use multiple sites across the entire cavity for both hydrophobic and hydrophilic substrate interactions. Thus, a bound substrate is accommodated in a protective environment in the interior of TF to prevent misfolding and aggregation. Various structural data further indicated that the protein is rather flexible, which seems essential for binding of the diverse set of substrates and for ribosome association. Bacterial ribosomes are bound by the monomeric form, while non-ribosome-bound TF shows a fast monomer–dimer equilibrium with a half-life of the dimer of ~1 s and a KD of ~1–2 μM. For the function of the TF dimer, opposing data have been reported. On the one hand, it was hypothesized that this dimer serves as an inactive storage form. On the other hand, it was found that such dimers contribute to the stabilization of unfolded substrate species.

In eukaryotes, chloroplasts are the only subcellular compartments that appear to contain molecular chaperones of the trigger factor family. Chloroplasts confer photoautotrophy to plants and algae. These organelles contain their own semi-autonomous genome, which encodes for approximately 60–100 proteins of all ~3000 chloroplast proteins. Most of the chloroplast-encoded proteins are major subunits of central protein complexes involved in gene expression and photosynthesis. Since chloroplasts are thought to have evolved from a photosynthetically active cyanobacterium more than a billion years ago, many components of the plastidic gene-expression machinery still resemble their cyanobacterial counterparts. For example, chloroplasts contain 70S ribosomes each consisting of a small 30S and a large 50S subunit. Consisting, many components of the chloroplast protein folding and quality control machinery are orthologous to the respective system in prokaryotes. Importantly, the chloroplast gene-expression apparatus also acquired novel mechanisms that are not found in cyanobacteria, which most likely serve the orchestrated expression of the nuclear, mitochondrial, and chloroplast genomes.

While bacterial TF has been studied extensively and might be the best understood molecular chaperone in the literature, no experimental evidence about the function and biophysical properties of plastidic trigger factor exists to date. Here, we describe biophysical and structural properties of plastidic trigger factor from Chlamydomonas (C. reinhardtii) and Arabidopsis (A. thaliana). Despite a high sequence variance among different chloroplast trigger factor species, small-angle X-ray scattering (SAXS) experiments indicated that the eukaryotic chaperones have an architecture strikingly similar to their bacterial counterpart. Yet, chloroplast trigger factors also show distinct molecular features that might have evolved to meet the functional requirements of the chloroplasts.

**Results and Discussion**

**The chloroplast trigger factor family displays low sequence conservation.** Genes orthologous to the bacterial TF can be found in all plastid-containing algae and plants, but are absent in non-photosynthetic eukaryotes. Thus, it has been postulated that algae and plants encode for a chloroplast isoform. To gain a first understanding of these chloroplast-localised chaperones, we compared plastidic trigger factor proteins from C. reinhardtii and A. thaliana with the isoform from E. coli. For simplicity and consistency with previous studies, the full-length chloroplast proteins will be abbreviated TIG1, and the E. coli form will be abbreviated EctF. Excluding the N-terminal chloroplast transit sequence of the TIG1 proteins, chloroplast and bacterial isoforms have a similar overall length and contain the three typical TF domains (i.e., N-terminal ribosome-binding domain, PPI domain, and C-terminal chaperone domain) (Fig. 1a and b). However, the amino acid composition displays only ~10% sequence identity and <30% sequence similarity between chloroplast TIG1 proteins and EctF. Similarly, C. reinhardtii and A. thaliana data share sequences of only 24% identity and 35% similarity (Fig. 1c).

Such low sequence conservation between two phyla is remarkable for molecular chaperones where at least some domains tend to be highly conserved among orthologues. Our phylogenetic analysis of various trigger factor sequences confirmed this high variability among orthologous forms from bacteria, algae, mosses and higher plants. Interestingly, TIG1 orthologues from diatoms and red algae show a closer homology to prokaryotic TFs, while TIG1 proteins from green algae, mosses, and higher plants fall into a separate clade. Within the green lineage, low sequence conservation is observed between chlorophyte and streptophyte TIG1 proteins (Fig. 1d). It might be speculated that the high variety among TIG1s from the green lineage constitutes an evolutionary adaptation of these proteins according to their task during protein biogenesis. Except for the moss P. patens, all investigated green algal and plant genomes encode only one full-length TIG1 species (Supplementary Table S1). Interestingly, mosses and higher plants seem to encode additional, truncated forms of a trigger factor. We propose to term these orthologous forms TIG2, as transit-peptide analyses predict a chloroplast localisation as well. TIG2 proteins seem to contain only the N-terminal ribosome binding domain. Since the N-terminal domain of bacterial TF is the major site for ribosome binding and exhibits some chaperone activity, an independent or distinct contribution of this truncated variant to protein biogenesis can be envisioned. It has been shown previously by size exclusion chromatography (SEC) and mass spectrometry of A. thaliana chloroplast extracts that TIG2s are expressed and that they co-migrate in high-molecular weight fractions together with TIG1 and plastid ribosomes. Phylogenetic comparison of C. reinhardtii and A. thaliana data reveals a clear separation of the two species (Supplementary Figure S1). TIG2 displays a closer relation to the full-length bacterial orthologues compared with TIG1. Thus, it is possible that, during evolution, two trigger factor orthologues were inherited from cyanobacteria and that TIG2 was subsequently lost in algae. Alternatively, TIG2 might have been acquired through horizontal gene transfer after separation of the algal and land-plant lineages.

**Most TIG1 is soluble but also thylakoid-associated.** For a biophysical comparison of chloroplast GrTIG1 and AtTIG1 with EctF, mature proteins lacking the N-terminal chloroplast transit peptide were cloned...
Figure 1. Evolutionary diversity of chloroplast trigger factor. (a) Alignment of trigger factor amino acid sequences from *Escherichia coli* (E.c.), *Arabidopsis thaliana* (A.t.), and *Chlamydomonas reinhardtii* (C.r.). Sequences were aligned by ClustalOmega and shaded using BoxShade (http://www.ch.embnet.org). Amino acids highlighted in black are perfectly conserved, similar residues are indicated by a grey background. Boxes indicate chloroplast transit-peptide cleavage sites as determined experimentally (green) and as predicted by TargetP and ChloroP (light red for *AtTIG1*)50, 51. Dark red box indicates putative ribosome-binding site and lines underneath the sequences indicate the three trigger factor domains according to the *E. coli* structure11. (b) *CrTIG1* and *AtTIG1* are predicted to comprise the typical three-domain organization of the N-terminal ribosome-binding domain (red), the PPIase domain (turquoise), and the C-terminal domain (blue). (c) Sequence homology comparison of *EcTF*, *CrTIG1*, and *AtTIG1* according to http://imed.med.ucm.es/Tools/sias.html. (d) Phylogram based on amino acid sequence alignments of trigger factor from gram-negative bacteria (*Escherichia coli*, *Vibrio cholerae*, *Thermotoga maritima*), cyanobacteria (*Nostoc* sp. strain PCC 7120, *Synechocystis* sp. PCC 6803, *Synechococcus* sp. PCC 7002, *Globergiella sulphuraria*, *C. merolae*, *P. tricornutum*), red algae (*Chlorella reinhardtii*, *C. subellipsosidea*, *Ochromonas* sp. *P. patens*), green algae (*O. sativa*, *B. distachyon*), and brown algae (*B. rapa*, *A. thaliana*, *C. grandiflora*).
for overexpression and affinity purification followed by subsequent SEC. This yielded highly pure and fully soluble proteins migrating with respective apparent molecular weights of 56 kDa (EcTIG1), 58 kDa (CrTIG1), and 57 kDa (AtTIG1), which is slightly larger than their theoretical molecular weights (i.e., 48.2 kDa (EcTF), 53.7 kDa (CrTIG1), and 53.2 kDa (AtTIG1)) (Fig. 2a). To test if the transit-peptide cleavage sites of CrTIG1 and AtTIG1 proteins were correctly predicted, purified proteins and cell lysates of C. reinhardtii and A. thaliana were separated by SDS-PAGE and analysed by immunoblot (Fig. 2b). Both endogenous and purified TIG1 migrated with the same velocity supporting the correct assumptions of transit-peptide cleavage sites.

To confirm that CrTIG1 is indeed a chloroplast-localised protein, we performed subcellular fractionation experiments. C. reinhardtii cells were fractionated into mitochondria, chloroplasts, stroma, thylakoids, and thylakoid-associated low-density membranes (LMs)28. The purity and degree of enrichment were tested with antibodies against chloroplast HSP70B, stromal CGE1, thylakoid membrane-associated CFI1, and mitochondrial carnitine palmityltransferase (CPT). CrTIG1 displayed a distribution similar to the chaperone HSP70B28 and was found mainly in the soluble stroma fraction of chloroplasts but is also associated to some extent with LMs and thylakoid membranes (Fig. 2c). Since a major fraction of bacterial TF is ribosome-bound9, the distribution of chloroplast ribosomes was visualised with an antibody directed against a protein of the 50 S subunit, PRPL1. Similar to CrTIG1 and HSP70B, ribosomes were enriched mostly in the stromal fraction but were also detectable in the thylakoid fraction. It has previously been reported that in diurnally growing C. reinhardtii cells, 20–30% of the chloroplast ribosome population is associated with thylakoids29. Here, less than 20% of the ribosomes were found in the thylakoid fractions. However, ribosomes may have dissociated from thylakoids during the long preparation process. For an independent assay of CrTIG1 and PRPL1 distribution, C. reinhardtii cells were examined by immunofluorescence. A hallmark of C. reinhardtii cells is the single cup-shaped chloroplast, which consumes most of the volume within the cell. The chloroplast contains a globular basal region with the pyrenoid and lobes extending apically from the basal region. Chloroplast localisation of CrTIG1 was confirmed by the typical cup-shape staining (Fig. 2d). Plastidic ribosomes were detected by fluorescence in situ hybridization (FISH) against ribosomal RNA and a specific PRPL1 antibody. Comparison of the suborganellar localisations of chloroplast ribosomes and CrTIG1 showed surprisingly different patterns. Ribosomes were mainly found within the globular basal region with highest concentrations proximal to the pyrenoid (highlighted by RbcL staining, Fig. 2d). CytG1 staining represented a patchy pattern distributed throughout the chloroplast. A stronger signal was detected in a region which showed little intensity of chloroplast ribosomes and may be outside or at the border of the chloroplast. While it cannot be ruled out that the antibody cross-reacts with cytosolic components, an alternative explanation is that some trigger factor protein may accumulate in a region which was previously shown to contain the cytosolic protein synthesis - and chloroplast import machinery30. The enrichment of trigger factor in chloroplasts was independently confirmed in A. thaliana lysates. Here, immunoblots clearly detect AtTIG1 in plastid fractions (Fig. 2e).

Chloroplast TIG1 proteins are unusually temperature-sensitive. The secondary structure of EcTF is predominantly α-helical for the N- and C-terminal domains, whereas the PPlase mainly contains β-sheets31,32. To gain insight into the secondary structures of the chloroplast TIG1 isoforms, both mature proteins were analysed by far-UV circular dichroism (CD) spectroscopy and compared with EcTF. Consistent with previous reports31,32, the far-UV CD spectrum of EcTF contained two pronounced minima at 208 nm and 222 nm, both typical for α-helical secondary structure. The spectra of both plastidic TIG1 proteins were comparable to that of EcTF, indicating that their overall secondary structures are similar (Fig. 3a). Secondary-structure estimation by the K2D2 algorithm33 indicates ~40% α-helix and ~13% β-sheet content for EcTF, close to the 43% α-helical and 16% β-sheet content determined from the respective crystal structure31. Both plastidic TIG1 proteins seem to contain a slightly higher proportion of α-helices (~44%), as indicated by a more intense negative ellipticity at 208 nm as compared with EcTF, and a similar content of β-sheets (12% and 13%, respectively). The data also confirm that all heterologously produced and purified proteins were folded and thus suitable for subsequent in vitro assays.

Thermal unfolding was studied by gradual heating of the proteins (Fig. 3a, right panels). The transition curves at 208 nm and 222 nm revealed qualitative differences between the bacterial and the plant proteins. For EcTF, a major unfolding transition was only observed at 222 nm, which started at ~30 °C and had a midpoint (where half of the population is folded and the other half unfolded) between 45 °C and 50 °C. This is consistent with another study34 using differential scanning calorimetry, which determined the unfolding midpoint of EcTF to be at 54 °C. This resistance against thermal unfolding was attributed to the dimeric form of EcTF34. For the chloroplast proteins, unfolding was traceable at both wavelengths, with unfolding starting just above 30 °C and featuring a local maximum in the ellipticity recorded at 222 nm at intermediate temperatures. The midpoints of the thermal unfolding of CrTIG1 and AtTIG1 were between 35°C and 40°C, which are far below the values for EcTF (Fig. 3a, right panels). Thus, even mild heat-stress conditions35 seem to cause partial unfolding of at least some domains. Such conformational changes may inactivate the chaperone or reprogram its function for other tasks during heat stress. For a more precise determination of the unfolding midpoint of CrTIG1, shifts of intrinsic tryptophan fluorescence upon thermal unfolding were recorded at wavelengths of 330 nm and 350 nm. We determined the
Figure 2. Intracellular localisation of TIG1. (a) EcTF, CrTIG1, and AtTIG1 were heterologously expressed in 
E. coli, purified via chitin affinity resins and size exclusion chromatography. 0.5 µg or 1 µg of each protein was 
separated by SDS-PAGE and stained with Coomassie. (b) 7.5 µg or 15 µg of soluble extracts from C. reinhardtii 
and A. thaliana was separated by SDS-PAGE next to 7.5 ng or 15 ng of purified TIG1 protein, transferred to 
nitrocellulose, and immunoblotted with antibodies against TIG1 (c). C. reinhardtii chloroplasts (CP) were isolated, 
lysed by hypo-osmotic shock, and separated into stroma (Str), low-density membranes (LM), and thylakoid 
membranes (Thyl). Mitochondria (Mito) were separated from the same strain. Whole cells (WC) and 7 µg of each 
fraction were separated by a 7.5–15% SDS-PAGE, transferred to nitrocellulose, and immunoblotted with antibodies 
against CrTIG1, chloroplast ribosomal protein PRPL1, HSP70B (stroma and membrane control), CF1β (thylakoid 
membrane associated), stromal CGE1, and mitochondrial carbonic anhydrase (CA). Note that LM might be over-
represented compared to thylakoids. (d) Images of C. reinhardtii cells, stained with antibodies directed against 
CrTIG1, RbcL, and PRPL1 (FITC, green) and a FISH probe against 70S ribosomes (red). The numbers of cells with the 
localisation patterns seen was 15 of 20 (75%) for CrTIG1, 16 of 20 (80%) for RbcL, 13 of 20 (65%) for PRPL1, 
and 20 of 20 (100%) for FISH. Antibody specificity is shown in Supplementary Figure S2. Scale bars represent 2 µm 
(e) 20 µg of soluble A. thaliana leaf extract or 20 µg of isolated chloroplasts was separated next to 7.5 ng purified 
AtTIG1 by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with antibodies against AtTIG1, Actin, 
and RbcL. Immunoblots in (b, c) and (e) were cropped to the respective size of the displayed bands.
unfolding midpoints $T_m$ in buffers having various ionic strengths of 0–300 mM KCl. With no additional salt present, CrTIG1 showed a $T_m$ of 35.6 °C, whereas $T_m$ was shifted up to 41.6 °C at 300 mM KCl (Fig. 3b). Thus, more physiological salt concentrations render the protein more robust against thermal unfolding.

We next tested if thermal unfolding could also be observed in living cells, which might lead to the accumulation of CrTIG1 in protein aggregates. For this purpose, *C. reinhardtii* cells were exposed to various temperatures of mild to severe heat stress (i.e., 35–45 °C), and insoluble aggregates were isolated (see Methods). Overall, little aggregation was observed in cells exposed to temperatures between room temperature and 37 °C. At non-lethal heat stress between 39 °C and 42 °C, increasing protein aggregation was observed with rising temperatures (Fig. 3c). The onset of protein aggregation at 39 °C was confirmed by examining the known heat-labile chloroplast protein rubisco activase (RCA1)36, which started to accumulate in aggregates at temperatures $\geq$39 °C. Interestingly, CrTIG1 was also detectable in the same fractions as RCA1 (Fig. 3d), while other chloroplast chaperones such as HSP90C or the co-chaperonin CPN20 predominantly accumulated in aggregates at higher temperatures. Thus, in contrast to bacterial TF and other molecular chaperones, chloroplast TIG1 is a remarkably heat-sensitive chaperone, which accumulates in aggregates even under relatively mild heat-stress conditions. Unlike many other chaperones, trigger factor is an ATP-independent chaperone. Thus, the observed heat sensitivity may serve as passive regulator to target this chaperone to protein aggregates which may serve as protective mechanism to rescue unfolded proteins from aggregates upon return to physiological growth conditions.
Chloroplast TIG1 is mainly monomeric. Several structural studies reported the occurrence of dimeric EcTF both in vitro and in vivo. To determine if chloroplast TIG1s show similar dimerization behaviours, purified TIG1 protein was separated by SEC. A prominent peak of EcTF protein eluted at a volume of 13.6 mL (Fig. 4, top panel), while CrTIG1 eluted in a minor peak at high molecular weight (12.1 mL) and a major peak at 14.5 mL (Fig. 4, middle panel), and AtTIG1 eluted at 14.1 mL with a small shoulder preceding the actual peak (Fig. 4, bottom panel). Since SEC elution profiles are not precise enough to determine the molecular weights of particles that are clearly nonspherical, as expected at least for EcTF, each fraction of the elution peak was examined online by right-angle static light scattering (RALS). The sloping curve of average molecular weight for EcTF resembles the molecular weight distribution of an earlier study with lower apparent masses of the protein at the peak borders and the highest value of 79 kDa in the centre of the peak (Fig. 4, top panel, Table S2). As stated in earlier studies, a heterogeneous population of monomeric and oligomeric EcTF seems to co-elute within this peak, which might represent a fast equilibrium of monomeric and oligomeric states. By contrast, both CrTIG1 and AtTIG1 were monodisperse, as indicated by the consistent molecular weight values within the major peak area (Fig. 4, centre and bottom panel) with polydispersity values of 1.001 and 1.002 ($M_w/M_n$), respectively. Further, elution profiles resulted in apparent molecular weight values close to their theoretical monomeric masses (Supplementary Table S2). Hence, at the physiological salt conditions used here, chloroplast TIG1s appeared to be present mainly in a monomeric form. In earlier proteomic studies, AtTIG1 migrated in native PAGE as expected for a molecular weight of the dimeric state. Dimeric species of TIG1 proteins cannot be excluded under certain conditions and in a native context; however, asymmetric and elongated proteins are known to migrate slower than globular proteins in native gels, which might explain the higher apparent molecular weight of AtTIG1 in the earlier studies. For our subsequent assays, it was essential that SEC fractions of CrTIG1 and AtTIG1 be highly monodisperse, making them ideally suited for three-dimensional bead modelling by SAXS.

**SAXS reveals a typical dragon-shape conformation of TIG1.** SAXS was used to examine if chloroplast TIG1s possess a similar overall shape as the bacterial isoform or if the eukaryotic chaperones evolved other structural
features. Initial static measurements were performed at different protein concentrations in three replicates to examine if the TIG1 proteins remain monodisperse also at higher protein concentrations. Importantly, SAXS parameters of the bacterial EcTF were very comparable to previously published datasets (Supplementary Figure S4). At the concentrations used here, the monomer–dimer equilibrium of EcTF was strongly shifted towards the dimeric state, although proteins are known to quickly fluctuate between monomeric and dimeric states even at high concentrations. Indeed, the values of the Porod volume indicate mainly dimeric proteins in the samples, but the molecular weight values derived from I(0) (i.e., the intensity at zero angle) point to a fast fluctuating population (Supplementary Table S3). Further, it has been reported that the radius of gyration (Rg) and the maximal particle size (Dmax) of EcTF decrease with increasing protein concentration, possibly because of a more densely packed dimer at higher concentrations compared with the monomer at lower concentrations. Consistently, the Rg and Dmax values determined here also decreased with increasing protein concentration, albeit to a lesser extent (Supplementary Table S3). Chloroplast CrTIG1 and AtTIG1 appeared predominantly as monomers assuming a predicted molecular weight of 53.7 kDa and 53.2 kDa, respectively. In contrast to EcTF, molar masses calculated from the I(0) values slightly increased with rising protein concentrations, most likely because of weak interparticle interference (Supplementary Tables S4 and S5). However, the high linearity of the Guinier plots speaks against aggregation of the samples (Fig. 5a and d, inset). To overcome this interference, low-range values at low concentrations and high-range values at high concentrations were merged for further processing. Both plastidic proteins were found to have an Rg value of ~38 Å, as determined by the Guinier plot and p(r) function. This very large Rg for a 53-kDa protein—as compared to an Rg value of 29.9 Å of globular BSA—indicates an elongated and non-globular shape of both proteins. This notion is supported by the asymmetric peak of the pair distance distribution functions of both TIG1s with a shoulder extending to a maximum particle size of 125 Å for CrTIG1 and 127 Å for AtTIG1, respectively (Fig. 5c and f).

All analysed trigger factor proteins showed an overall similar bimodal pattern in Kratky plots with a dominant first peak followed by a distinct small peak indicating well-folded and multi-domain proteins. In addition, pronounced intrinsic flexibilities are indicated by the behaviour of the plots at high s-range, which do not converge to the abscissa. The Kratky plot of EcTF displays a slight shift compared with both plastidic TIG1 proteins, indicating a more rigid and compact conformation due to the dimeric state (Fig. 5b,e and Supplementary Figure S4).

To verify our results regarding the monomeric state obtained from static SAXS measurements, we further performed online SEC coupled to SAXS detection (HPLC-SAXS). Consistent with the static assays, only minor amounts of putative dimer populations were detectable in the CrTIG1 and AtTIG1 samples, as indicated by the slight tailing of the monomeric peak fraction (Fig. 6a and d). To simplify our analysis of the chloroplast proteins,
these higher molecular weight fractions were neglected in all subsequent steps. Thus, only frames within the peak section of high quality and constant $R_g$ were averaged for further analysis (Fig. 6a,d and Supplementary Figure S5, grey shaded area). The molecular weight was determined using multiple, concentration-independent, methods. These seem to overestimate the mass of the chloroplast TIG1s by up to 17% (Table 1), which might be attributed to traces of dimers or to the influence of capillary fouling on scattering42. However, the masses determined by RALS strongly supported predominantly monomeric states. Importantly, the calculated $R_g$ (34.4 Å for CrTIG1, 35.5 Å for AtTIG1) and $D_{\text{max}}$ (122 Å for CrTIG1, 124 Å for AtTIG1) values were comparable to the results from static measurement, which allowed us to complementarily process data from both approaches for further modelling.

Figure 6. SAXS data from HPLC-SAXS experiments of trigger factors. Small-angle X-ray scattering data were collected by size-exclusion high-performance liquid chromatography with a ENrich SEC 650 column online with small-angle X-ray scattering (HPLC-SAXS) with 400 µg of the respective protein. (a) and (d) Elution profiles of SEC-SAXS runs, represented by $I(0)$ and $R_g$ determined by AUTORG for each frame. Highlighted in grey are the frames used for averaging. (b) and (e) Experimental SAXS profiles from the averaged HPLC-SAXS frames of CrTIG1 and AtTIG1 are indicated by small circles. Respectively, red curves represent the fit by GNOM, blue curve the CRYSOl fit of the RaptorX models, and grey curves the theoretic scattering of final DAMMIN model. Inset: Guinier plot of $\ln I(s)$ versus $s^{-2}$ obtained from AUTORG. Data points used by AUTORG are labelled in blue. (c) and (f) Corresponding $p(r)$ function as calculated from the experimental scattering curves using GNOM. “au” is arbitrary units. For SAXS data on EcTF and the FoXS fit, see Supplementary Figure S5.

Table 1. Structural parameters derived from static and HPLC-SAXS experiments. For static measurements, scattering curves from different concentrations were merged. For HPLC-SAXS, parameters were determined for the averaged peak scattering. Molecular weights (MW) were estimated using Porod-volume/1.7, the DAMMIN volume/2, and using the datmow tool included in the ATSAS suite.
All plastidic TIG1 SAXS datasets were applied for *ab initio* modelling of low-resolution bead envelopes. Modelling of *E. coli* TF was not pursued with our dataset since the heterogeneous population of dimeric and monomeric species complicates modelling and different conformations of purified *E. coli* TF proteins were comprehensively studied before. With the static SAXS measurements, more defined scattering at a higher *s*-range was obtained because of a higher protein concentration in the sample. Hence, *ab initio* modelling was pursued with the GASBOR algorithm, which allows a chained assembly of dummy residues corresponding to the number of amino acids. The models obtained fitted the experimental data with *χ*² values of 0.81 for *C. reinhardtii* and 0.69 for *A. thaliana* (Supplementary Table S6). To confirm that the minor traces of putative dimeric species did not significantly influence the models, data were processed in symmetry P1 (no symmetry) and P2 (dimer). The P2 symmetry could be excluded because of the resulting high *χ*² values of these models (data not shown) and hence supported the fact that our models were truly based on the monomeric molecules. For the HPLC-SAXS dataset, *ab initio* modelling was performed with DAMMIF. Consistent with the modelling of the static SAXS data, *χ*² values of 0.72 for *C. reinhardtii* and 0.79 for *A. thaliana* (Supplementary Table S6) were gained for these low-resolution structures.

The low-resolution bead models of *C. reinhardtii* and *A. thaliana* proteins resulting from both datasets clearly resemble the conformation of the bacterial TF with a dragon-like structure containing head, tail and arms (Fig. 7). Interestingly, groove-like structures are visible along the middle domain (Supplementary Figure S6), which could constitute putative binding sites for peptide stretches of plastidic TIG1 substrates.

Next, we attempted to fit high-resolution models into the SAXS-based *ab initio* shapes. Since, to our knowledge, no structural data exist for any plastid TF to date, predicted models were calculated with the RaptorX and SAXSTER algorithms on the basis of various structural data of exclusively full-length bacterial isoforms. Different high-resolution structures of TF exist for dimeric apo *E. coli* TF complexes (X-ray structure, PDB entry 1w26) and monomeric TF associated with different fragments of the PhoA (NMR structures, PDB entries 2mly/2mlz) or the ribosome (PDB entry 2vhr). Further, crystal structures of dimeric TF from *V. cholerae* (PDB entry 1114) and *T. maritima* are available. For the latter, both full-length structures with and
without a substrate are monomeric (X-ray structures, PDB entries 3gtv/gto8). Different bacterial TF species show various domain orientations50. For example, both the crystal structure and NMR structural analyses reveal different orientations of the head domain among EcTF51, VcTF52, and TmTF38. For VcTF, both domains constituting the arms and the tail show a twist, which is not seen for the other isoforms50. Psi-Blast searches with sequences of CrTIG1 and AtTIG1 resulted in similar matches between the plastidic TIG1 proteins and the bacterial TFs (Supplementary Table S7). The best coverage for CrTIG1 and AtTIG1 was reached with the full-length NMR and cryo-EM structures of EcTF (PDB entry 2mlx and 2vry) (Supplementary Table S7). Our predicted models were validated with CRYSOL and superimposed with the bead models. Again, the models that gave the best $\chi^2$ values in CRYSOL were the predicted models from RaptorX based on 2mlx. Importantly, not all high-resolution models fitted with same quality. Superimposing the models based on VcTF and TmTF to the SAXS data was not successful as judged by their high $\chi^2$ scores (>25–55, data not shown). To test if we could further improve the domain agreement of our positive high models within the SAXS-based bead models, SREFLEX was applied, which examines the flexibility of models. Generally, this refinement improved the $\chi^2$ values, albeit with a different outcome. For CrTIG1, $\chi^2$ values were slightly improved from 10.4 to 6.2. SREFLEX Refinement of CrTIG1 might have been less than optimal since the orientations of some $\alpha$-helices within the central body domain were severely altered (Fig. 7a). In contrast, the refinement of AtTIG1 high-resolution models improved $\chi^2$ values significantly from 57.2 to 3.3 (Supplementary Table S6) with only mild rearrangements within the domains (Fig. 7c). As shown in Figs 5 and 6, two independent evaluations of our high-resolution models by CRYSOL and a simulation of theoretical scattering and fitting to our experimental data by FoXS resulted in close fits of the models to the SAXS data. Thus, it can be assumed that the E. coli-based models closely describe the overall shape and domain position of the plastidic TIG1 proteins. However, none of the bacteria-derived models tested resulted in a complete match to the SAXS shapes obtained from the static and HPLC-RALS SAXS measurements from the static and HPLC-RALS SAXS measurements and subsequent ab initio modelling revealed the typical dragon-shape of the molecule as known from bacterial TF containing a head, a tail, and two arms. This trigger-factor-like shape was confirmed by independent high-resolution models, which fitted well to the SAXS data. However, the plastoplast TIG1 models indicate unique domain arrangements and intrinsic conformations, thus suggesting that plastidic TIG1s have structurally diverged from their bacterial orthologues. Given the ubiquitous action of bacterial TF on most newly-synthesized proteins, plastidic TIG1s – and maybe even the truncated version TIG2 – have specifically adapted to serve the maintenance of protein homeostasis in chloroplasts. It will be interesting to see how chloroplasts are the only compartment of eukaryotic cells that conserved these trigger factor chaperones. Future studies are also required to determine the detailed function and substrate specificity of these chaperones in chloroplasts.

Conclusions
In conclusion, we present here the first description of two eukaryotic chloroplast-localised molecular chaperones of the trigger factor family. Both plastidic TIG1 orthologues are mainly monomeric, as shown independently by RALS and SAXS for the purified proteins. The most surprising finding was the high propensity of plastidic TIG1 for thermal unfolding, which might perform a specific task during heat exposure. Independent static and HPLC SAXS measurements and subsequent ab initio modelling revealed the typical dragon-shape of the molecule as known from bacterial TF containing a head, a tail, and two arms. This trigger-factor-like shape was confirmed by independent high-resolution models, which fitted well to the SAXS data. However, the plastoplast TIG1 models indicate unique domain arrangements and intrinsic conformations, thus suggesting that plastidic TIG1s have structurally diverged from their bacterial orthologues. Given the ubiquitous action of bacterial TF on most newly-synthesized proteins, plastidic TIG1s – and maybe even the truncated version TIG2 – have specifically adapted to serve the maintenance of protein homeostasis in chloroplasts. It will be interesting to see how chloroplasts are the only compartment of eukaryotic cells that conserved these trigger factor chaperones. Future studies are also required to determine the detailed function and substrate specificity of these chaperones in chloroplasts.

Methods
Cells and Culture Conditions. Most experiments were conducted with the C. reinhardtii strain cw15 CF18548. Microscopy was done with strain cw15–325 (cw d, mt $^+$), with TargetP and ChloroP50, 51 predicted the transit peptide cleavage site QVC/A. Mature TIG1 both TF and TF to the SAXS data was not success-

Cloning, production and purification of the trigger factor species. For CrTIG1 both TargetP and ChloroP50, 51 predicted the transit peptide cleavage site QVC/A. Mature C. reinhard-

TIG1 protein (lacking the putative N-terminal 67 amino-acid transit peptide) was created by site directed mutagenesis of pFW13 resulting in pFW115. For AtTIG1, the predicted cleavage site was RVS/S. Mature A. thaliana TIG1 with the putative N-terminal 27 amino-acid transit peptide, was ampli-

ified by PCR from cDNA with the primers (5′-GGGCGATATGCCTCGTCCTTCAATCAG-3′) and (5′-CCCCGGATATCCCAACGAGTGATGTATTGAATC-3′) and cloned with NdeI and BamHI into pTyb21 (NEB) resulting pFW14. Since the cleavage site was predicted incorrectly, the cleavage site RLF/A was reassigned based on the alignment of AftIG1 with CrTIG1. For correction of the mature protein, 49 amino acids were removed from the N-terminal sequence with oligos 5′-GGAAGAGCCTCATTAGCTCGGTTCCTTCAATCAGATGTGTGCACTAGTTGTTGGGAGGTGGTGAAGAAGAACATAATCAGAGCCCTTTGAGACATGTCTTGTTGTCCTAGAAAAATTTGGAATTTTATGTCACTAGTGGTGAATTTTATGAGTATCCTCCGATATCCCAACGAGTGATGTATTGAATC-3′ by site directed mutagenesis resulting in pFW136. The TF sequence from the EcoRI fragment of pFW136 was amplified by PCR from DH5alpha genomic DNA with primers (5′-GGGCGATATGCCTCGTCCTTCAATCAG-3′) and (5′-CCCCGGATATCCCAACGAGTGATGTATTGAATC-3′) and cloned with NdeI and BamHI into pTyb21 (NEB) resulting pFW142. For heterologous protein production pFW115, pFW136 and pFW142 were synthesized in E. coli.
coli ER2566 and purified via chitin affinity resins according to the manufacturer’s instructions (NEB). All purified proteins were concentrated with Amicon Ultra-15 concentrator (Merck Millipore, Darmstadt) and purified over a Superdex 200 SEC column (GE healthcare) in 10 mM Tris pH 7.5 and 10 mM KCl buffer. For antisera production, rabbits were immunized with C/TIG1 or A/TIG1.

Phylogenetic analyses and comparison of sequence similarity and identity. Sequences were derived from http://phytozome.jgi.doe.gov, NCBI and http://www.uniprot.org. Phylogenetic analyses were performed with sequences comprising the mature sequences lacking predicted transit peptides using the Phylogeny.fr pipeline implementing algorithms T-Coffee, BioNJ, and TreeDyn setting bootstraps values to 1000. Sequence similarity and identity was determined by http://imed.med.ucm.es/Tools/sias.html.

Immunofluorescence microscopy. Cell fixation and staining was done as published before. Primary antibodies were against C/TIG1, RbCL, and PRPL1 in dilutions of 1:300, 1:2,000, and 1:1,000, respectively. The secondary fluorescein isothiocyanate-labeled antibody (Sigma-Aldrich) was applied in a 1:200 dilution. FISH probes were prepared according to . After incubation with the secondary antibody, slides were washed in phosphate-buffered saline, and a drop of mounting solution containing DAPI (Vectashield; Vector Laboratories) was applied at the centre of each slide. Samples were analysed with a Leica TCS SP5 II confocal laser-scanning microscope (514-nm excitation and 500-, 592-, or 598–657 nm detection).

Circular dichroism spectroscopy. CD spectra were recorded on a Chirascan-plus (Applied Photophysics) spectropolarimeter. All proteins were measured at a concentration of 0.1 mg/mL in 10 mM KCl, 10 mM Tris, pH 7.5 buffer. At fixed temperatures (20°C), five scans between 190 nm and 280 nm were recorded with an optical pathlength of 0.1 cm, a step size of 1 nm, and a digital integration time of 1 s. All spectra were baseline-corrected. At the two minima at 222 nm and 208 nm, temperature scans were recorded from 20°C to 94°C at a heating rate of 1°C/min and a digital integration time of 4 s.

Thermal stability/melting point determination using Differential Scanning Fluorimetry. Protein thermal stability was measured at 5 mg/mL protein concentration in different buffer compositions using the Prometheus NT.48 (NanoTemper Technologies). Thermal unfolding was performed in nanoDSF grade high-sensitivity glass capillaries (NanoTemper Technologies) with a heating rate of 1°C/min over a temperature range of 20–95°C. Protein-unfolding midpoints (Tm) were calculated from the first derivative of the ratio of intrinsic protein fluorescence emission intensities at 330 nm and 350 nm.

Analysis of protein aggregates. Ch. reinhardtii cells were grown to logarithmic phase. For each sample, 3 × 10^8 cells were harvested and resuspended in 50 mL of fresh medium at the respective temperatures. After 45 min, cell pellets were harvested quickly and chilled on ice. Aggregate preparations were done as published previously. In brief, cell pellets were resuspended in lysis buffer (20 mM NaPi pH 6.8, 10 mM EDTA, 0.25x proteinase inhibitor (Roche, cOmplete-EDTA-free)) and lysed by sonication (Bandelin Sonopuls UW 2200, Berlin). Cell debris was removed by centrifugation at 500 g, and supernatants were adjusted to the same protein concentrations. As input control, “Total lysate” aliquots were removed and supplemented with 1 volume of sample buffer (125 mM Tris–HCl, pH 6.8, 20% glycerol, 4% SDS, 100 mM EDTA, and 0.005% bromophenol blue). From remaining lysates, protein aggregates were isolated by repetitive centrifugation at 19,000 g and washes with 20 mM sodium phosphate pH 6.8 and 2% NP-40. The final pellet was dissolved in 6 M urea and supplemented with 1 volume of sample buffer before separation on SDS-PAGE, transfer to nitrocellulose membrane, and immunoblotting.

RALS measurements. SEC coupled to RALS was performed on an OMNISEC system (Malvern Instruments, Worcestershire, UK) using a Superdex 200 Increase 10/300 SEC column (GE Healthcare). Prior to each run, the system was extensively equilibrated in running buffer (20 mM Tris, pH 7.5, 150 mM KCl). Protein concentrations were determined from online refractive index (RI) measurements assuming a d_n = 1.336 g/mL and corresponds to a q-range of 0.025–5 nm^-1. For static measurements, five to eight different protein concentrations were measured with 1 s exposure times per frame and 10 frames per concentration (n = 3) at 20°C. Scattering by proteins samples were measured in 20 mM Tris buffer, pH 7.5 supplemented with 150 mM KCl. Scattering by the corresponding buffers was measured before and after one run, averaged and subtracted from the protein scattering. BSA standards were used to calibrate the I(0) values and the scattering of pure water was used to calibrate the intensity to absolute units. Prior to measurements, protein concentrations were determined from absorption at 280 nm (A_280) using a NanoDrop One photometer (Thermo Scientific) and the corresponding molar coefficient calculated with ProtParam (www.expasy.ch).

For SEC high-performance liquid chromatography/small-angle X-ray scattering (HPLC-SAXS), an online HPLC system (Shimadzu, Japan) connected to the sample changer of the BM29 was used. Per run, 400 µg protein (50 µL with 8 mg/mL protein concentration) was injected onto an ERich SEC 650 10 mm × 300 mm column (BioRad) and separated at a flow rate of 0.75 mL/min at room temperature. 1500 frames with an exposure time of 1.5 s per frame were collected per run.

X-ray scattering experiments and data analysis. Small-angle X-ray scattering data were collected on the BM29 beamline at the European Synchrotron Radiation Facility (ESRF, Grenoble) with a Pilatus 1 M detector (16.9 cm × 17.9 cm) at a wavelength of 0.9919 Å (12.5 keV) and a sample-detector distance of 2.867 m corresponding to a q-range of 0.025–5 nm^-1. For static measurements, five to eight different protein concentrations were measured with 1 s exposure times per frame and 10 frames per concentration (n = 3) at 20°C. Scattering by proteins samples were measured in 20 mM Tris buffer, pH 7.5 supplemented with 150 mM KCl. Scattering by the corresponding buffers was measured before and after one run, averaged and subtracted from the protein scattering. BSA standards were used to calibrate the I(0) values and the scattering of pure water was used to calibrate the intensity to absolute units. Prior to measurements, protein concentrations were determined from absorption at 280 nm (ε_280) using a NanoDrop One photometer (Thermo Scientific) and the corresponding molar coefficient calculated with ProtParam (www.expasy.ch).
The data processing was done with the ATSAS 2.7.2 program package\(^{44,45}\). The forward scattering \(I(0)\) and the radius of gyration \(R_g\) were evaluated with PRIMUS\(^{46}\) using the Guinier approximation assuming that for a spherical particles at very small angles \((s < 1.3(R_g))\) the intensity is represented by \(I(s) = I(0) \exp(-s^2R_g^2/3)\). The distance distribution function \(p(r)\) and the maximum particle dimension \((D_{max})\) were obtained using GNOM\(^{47}\).

Frames collected from static measurements were checked for radiation damage before averaging and buffer subtraction. Scattering curves from the concentration series were investigated individually for indications of aggregation and particle attraction or repulsion. Curves affected were excluded from further analysis. For the characterization of the oligomerization state, scattering curves (from \(n = 3\)) with same protein concentration were scaled and merged. For \textit{ab initio} modelling, frames from different concentrations were merged. Frames of the HPLC-SAXS, which were automatically processed by EDNA, were checked and inspected with HDFView. Frames with a consistent \(R_g\) from the peak scattering intensity and good quality were scaled manually and averaged to yield a single frame. Buffer subtraction was applied using buffer frames before or after the protein peak avoiding over-subtraction with frames from the aggregate peak.

A first insight into the overall shape of the TIG1 proteins was given by the \textit{AMBI METER} tool. These models yielded a crude shape topology, which was further refined and supported by \textit{ab initio} modelling. For the latter, 10–20 models were generated with GASBOR\(^{48}\) or DAMMIF\(^{49}\) and then averaged with DAMAVER\(^{50}\). As a last refinement step, the \textit{damstart} model generated with DAMAVER was refined with one cycle in DAMMIN. Protein structure modelling was performed with the SAXSTER server\(^{71}\) on the basis of all available full-length TF structures (\textit{i.e.} from \textit{E. coli}, \textit{T. maritima}, and \textit{V. cholerae}) as templates or RaptorX\(^{37}\) algorithm based on the \textit{EcTF} structure. Superposition of \textit{ab initio} models derived from SAXS data and 3D models were calculated with SUPCOMB\(^{72}\) or \textit{SUPALM}\(^{73}\). Fits between the SAXS data and the structures were evaluated with \textit{CRYSOL}\(^{74}\) and \textit{FoXS}\(^{75}\) and superposed with the bead models using \textit{SUPALM}\(^{71}\). The models were improved using \textit{SREFLEX} in rigid or flexible mode with domain partition\(^{76}\). To this end, sequences regions of the domains were determined from the RaptorX model in accordance with the domain annotation of the available TF structures (Supplementary Table S8). Additionally, theoretical scattering profiles from the models were generated and fitted against the experimental data with the \textit{FoXS} Server\(^{75}\).

**Miscellaneous.** Chloroplast and mitochondria were isolated from \textit{C. reinhardtii} cells as described earlier\(^{57}\). SDS-PAGE was performed as published\(^{57}\). Antibodies described earlier were against HSP70\(^{58}\), CGE\(^{76}\), HSP90\(^{26}\), mitochondrial carbonic anhydrase\(^{80}\), CytP\(^{80}\) and CFI\(^{94}\). Antibody purification was described earlier\(^{58}\).

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Author Contributions
F.R. purified proteins, conducted C.D., RALS, and SAXS experiments and analysed SAXS data; Y.C. helped with SAXS measurements, performed differential scanning fluorimetry, analysed SAXS data and wrote parts of the manuscript; M.R. contributed immunofluorescence and comparison of purified proteins with cell lysates; K.G. purified the proteins; S.K. provided instruments for C.D. and SEC-RALS measurements and helped with data analysis; C.R.D.L. coordinated SAXS analysis and helped with SAXS data evaluation; F.W. designed experiments, prepared the figures and wrote the manuscript.

Additional Information
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