Running Title: cpSRP54 function in chloroplasts

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Quantitative proteomics of a chloroplast SRP54 sorting mutant and its genetic interactions with CLPC1 in Arabidopsis thaliana

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Abstract cpSRP54 is involved in co- and post-translational sorting of thylakoid proteins. The Arabidopsis cpSRP54 null mutant, ffc1-2, is pale green with delayed development. Western blot analysis of individual leaves showed that the SRP sorting pathway, but not the SecYE translocon, was strongly down-regulated with progressive leaf development in both wild-type and ffc1-2. To further understand the impact of cpSRP54 deletion, a quantitative comparison between ffc2-1 was carried out for total leaf proteomes of young seedlings and for chloroplast proteomes of fully developed leaves, using stable isotope labeling (iTRAQ and ICAT) and 2-dimensional gels. This showed that cpSRP54 deletion led to a change in LHC composition, increase of PsbS, and a decreased Photosystem I/II ratio. Moreover, the cpSRP54 deletion led in young leaves to up-regulation of thylakoid proteases and stromal chaperones, including ClpC. In contrast, the stromal protein homeostasis machinery returned to wild-type levels in mature leaves, consistent with the developmental down-regulation of the SRP pathway. A differential response between young and mature leaves was also found in carbon metabolism, with an up-regulation of the Calvin cycle and the photorespiratory pathway in peroxisomes and mitochondria in young leaves but not in old leaves. In contrast, the Calvin cycle was down-regulated in mature leaves, likely to adjust to the reduced capacity of the light reaction, while reactive oxygen species defense proteins were up-regulated. The significance of ClpC up-regulation was confirmed through the generation of an ffc2-1 x clpc1 double mutant. This mutant was seedling-lethal under autotrophic conditions, but could be partially rescued under heterotrophic conditions.
INTRODUCTION

Chloroplasts are essential for plant growth and development. Nuclear-encoded chloroplast proteins are imported into the chloroplast via the Tic/Toc complexes (Jarvis and Robinson, 2004; Soll and Schleiff, 2004; Kessler and Schnell, 2006), followed by processing, folding and assembly by various chaperone systems. Proteins destined for the thylakoid membrane or thylakoid lumen are targeted by four different sorting pathways that can be distinguished based on their energy requirements and protein components (Mori and Cline, 2001; Schunemann, 2007; Jarvis, 2008).

One of these post-translational pathways is the Signal Recognition Particle (SRP) pathway which targets specific members of Light Harvesting Complex (LHC) protein family to the thylakoid membrane (Eichacker and Henry, 2001; Schunemann, 2004). These LHC proteins have a partially conserved 18 amino acid sequence between the 2nd and 3rd transmembrane domain (TMD) – this was named the L18 motif (DeLille et al., 2000; Tu et al., 2000). The SRP pathway involves cpSRP54, cpSRP43, cpFtsY and the integral thylakoid membrane protein Alb3 (Li et al., 1995; Sundberg et al., 1997; Schunemann et al., 1998; Kogata et al., 1999), reviewed in (Schunemann, 2004). It was shown that the L18 motif is required for interaction with cpSRP43, while cpSRP54 interacts with the hydrophobic TMDs of the LHCs and ankyrin repeat domains of cpSRP43. In light of the conserved co-translational sorting function of the SRP pathway in bacteria and the ER, the discovery of the post-translational SRP pathway for nuclear-encoded thylakoid proteins was surprising (for discussion (Pool, 2005)). SRP43 appears to be unique for organisms with LHC proteins. cpSRP54 has specific modifications that also allow it to interact with cpSRP43 and function without the SRP-RNA moiety observed in bacteria and in the cytosol of eukaryotes (Jaru-Ampornpan et al., 2007; Chandrasekar et al., 2008).

In contrast to post-translational targeting, less is known about the sorting and insertion of the 40 plastid-encoded integral thylakoid membrane proteins. It is generally believed that integral thylakoid proteins are synthesized on 70S ribosomes attached to the thylakoid membrane, possibly directly docking on the SecY/E translocon. cpSRP54 also associates with 70S ribosomes (Franklin and Hoffman, 1993; Li et al., 1995; Schunemann et al., 1998), suggesting a role for cpSRP54, but not cpSRP43, in sorting of chloroplast-encoded proteins. Indeed, cpSRP54, but not cpSRP43, was shown to interact tightly but transiently with the first
TMD of the D1 nascent chain (Nilsson et al., 1999; Nilsson and van Wijk, 2002). Moreover, during co-translational insertion, the D1 nascent chain interacts with cpSecY (Zhang et al., 2001). In contrast, chloroplast-encoded Cyt f, with cleavable lumenal transit peptide, was shown to be co-translationally inserted into the membrane requiring cpSecA, the Sec translocon and ATP, as well as a functional ITP (Rohl and van Wijk, 2001).

cpSRP43, cpSRP54 and cpFtsY are each single copy genes in the Arabidopsis genome. Homozygous single and double mutants in cpSRP43/54 and cpSRP54/cpFtsY can be maintained and propagated on soil. Two mutant lines affecting cpSRP54 have been described: a dominant co-suppressor in which cpSRP54 protein levels is reduced by 70-93 % (Pilgrim et al., 1998) and a null mutant (ffc1-2) (Amin et al., 1999). The ffc1-2 mutant shows both pale cotyledons and pale true leaves in particular when plants are very young. Analysis of the Arabidopsis cpSRP43 null mutant (chaos) (Amin et al., 1999; Klimyuk et al., 1999), cpFstY mutants (Durrett et al., 2006; Tzvetkova-Chevolleau et al., 2007), the Alb3 null mutant (Sundberg et al., 1997) and double mutants chaos x ffc1-2 (Hutin et al., 2002) and cpftsy x ffc1-2 (Tzvetkova-Chevolleau et al., 2007) resulted in various conclusions and models. Collectively these studies showed that: i) the thylakoid protein translocon ALB3 is likely essential for LHC insertion, ii) cpSRP43 can target LHC proteins to ALB3 in absence of cpSRP54 and cpFtsY, albeit with reduced efficiency, iii) complex formation of cpSRP54 and cpSRP43 likely prevents cpSRP43 from targeting proteins independent of cpFtsY, as evidenced by suppression of the strong cpftsy phenotype when crossed with ffc1-2, iv) presence of cpSRP43 is not absolutely required for targeting of LHC proteins, and v) cpSRP54, together with cpFtsY, stimulates targeting chloroplast-encoded thylakoid membrane proteins, but that this co-translational pathway is not essential. In disagreement with these in vivo observations, in vitro experiments indicated that cpSRP54, 43 and cpFtsY are each strictly required for targeting of LHC (Schunemann, 2004).

While the primary functions of cpSRP54 in intra-plastid protein sorting are clear, little is known about the secondary effects of the loss of cpSRP54, including the plastid gene expression machinery, chaperones and metabolic enzymes. Moreover, the SRP mutant analyses suggest alternative and compensatory pathways and factors that are so far unknown (Pilgrim et al., 1998; Amin et al., 1999; Hutin et al., 2002; Tzvetkova-Chevolleau et al., 2007). This study aims to address these aspects. To that end, we developed a mass spectrometry (MS) based strategy for quantitative comparative proteomics of very young and mature Arabidopsis ffc1-2 and wild-type
(wt) seedlings. Isobaric stable isotope labeling (iTRAQ), isotope coded affinity tags (ICAT) and 2-dimensional gel electrophoresis (2DE) were used, since they are complementary techniques. This approach can also be applied for the analysis of other mutant seedlings (with or without developmental delays) and provides in particular an attractive general method for the analysis of chloroplast mutants, especially if these mutants have strong phenotypes from which it will be difficult to isolate significant amounts of intact chloroplasts. To further understand the chloroplast proteome homeostasis network and to follow-up on the proteomics results, we generated an ffc2-1 x clpc1 double mutant. These findings are integrated in the context of chloroplast biogenesis, development and metabolism.

RESULTS

The ffc1-2 mutant is delayed in development irrespective of growth light intensity

The ffc1-2 mutant was generated by EMS mutagenesis and is devoid of cpSRP54 transcript and protein (Amin et al., 1999). Throughout the remainder of this article we refer to ffc1-2 as ffc. When ffc was grown on agar plates under continuous moderate light (70 µmol photons.m\(^{-2}\).s\(^{-1}\)), young seedlings showed yellow first leaves, that subsequently became green (Amin et al., 1999). However, when ffc was grown on soil with a light dark period at 300 µmol photons.m\(^{-2}\).s\(^{-1}\) plants remained pale green with reduced rosette diameter (Hutin et al., 2002). To better characterize plant growth and development of ffc, wild-type (wt) and ffc were grown on soil under four different light intensities and their visible phenotype and rate of leaf development and rosette diameter were carefully monitored (Fig. 1). Plants were sown directly on soil without replanting to minimize biological variation. Figure 1 shows wt and ffc plants 33 days after planting when grown under four different light regimes (note that the magnification is not the same for each plant). ffc plants were always paler, with less leaves and a 40-60% reduction in rosette diameter (Fig. 1). Bolting was delayed by 25% under each light regime but occurred after formation of the same number of leaves as in wt indicating that loss of cpSRP54 does result in a delay in development (data not show). Therefore, to understand the role of cpSRP54 in chloroplast and leaf development, primary cpSRP54 effects and secondary developmental effects must be distinguished.
Accumulation of the chloroplast SRP targeting machinery, the LHCII family and the D1 and D2 protein during leaf development

The chloroplast sorting machinery is particularly important during early leaf and chloroplast development when protein synthesis and import rates are high. We postulated that the plastid protein sorting machinery might be down-regulated with progressive leaf development. There has been no systematic investigation on the response of the chloroplast sorting pathway in the ffc mutant. However, we note that cpSecY levels were determined in total leaf extracts of 10 and 24 days old ffc and wt plants grown on agar plates (with vitamin mix) under continuous moderate light; this showed that cpSRP43 was not affected, while cpSecY levels were somewhat increased in ffc (Amin et al., 1999). In order to separate changes in protein accumulation due to the ffc mutation from those naturally occurring throughout development, we used the developmental stage of both wt and ffc, and not the age of plant material as the basis for selecting leaf material for analysis. Developmental stages used in this study were as defined in (Boyes et al., 2001). Specifically, the developmental stage of plants in the vegetative stage are determined by the number of leaves, which is indicated by the number after the digit, e.g. stage 1.4 is a young seedling with 4 rosette leaves of at least 1 mm. To monitor the direct effects to the protein sorting machinery along with development in both ffc and wt, we first determined the chlorophyll content and total protein content of the cotyledons and leaves 1&2, 3&4, 7&8, 11&12 at developmental stages 1.4, 1.8, 1.12 and 1.16 for each leaf pair. Protein to chlorophyll ratios in each leaf decreased strongly (upto 50-fold) during development, reflecting assembly of the photosynthetic apparatus and accumulation of chlorophyll in the thylakoid membrane; ffc leaves trailed in their chlorophyll accumulation (Suppl. Fig.1). This is consistent with a 60% reduction of chlorophyll levels observed for leaf rosettes of ffc plants as compared to same aged wild-type plants (Hutin et al., 2002).

Accumulation levels of the cpSRP pathway proteins, cpSRP54, cpSRP43, cpFtsY, and the thylakoid protein translocons, Alb3 and cpSecY,E were determined for these separate leaf pairs (Fig. 2A,B,C) and cotyledons (not shown) at different developmental plant stages. This showed that accumulation levels of SRP pathway components (cpFtsY, cpSRP43, cpSRP54, ALB3) were strongly reduced with progressive leaf development (Fig. 2A), whereas cpSecY (Fig. 2B) and cpSecE (Fig. 2C) levels were relatively constant. Comparing ffc with wt showed that, when seedlings of the same developmental stage were compared, levels of each of these
sorting components were similar (except for cpSRP54 which was absent in ffc) (Fig. 2A,B,C). This provides additional evidence that comparison of wt and ffc leaves of similar development stage, rather than the same age, will best determine direct effects of cpSRP54 deletion.

Silver stained one-dimensional (1-D) SDS-PAGE of whole leaf extracts of the leaf pairs at the different developmental stages showed that the overall accumulation levels of the family of major LHCII proteins (with a molecular mass of 25-27 kDa) became more abundant during leaf maturation (Fig. 2D - upper panel), as expected from previous studies. The LHCII levels were always lower in ffc, as compared to wt leaves. As an example, the profiles for leaves 3&4 and 7&8 at the stages 1.4, 1.8, 1.12, 1.16 are shown (Fig. 2D – upper panel). The levels of chloroplast-encoded D1 and D2 thylakoid membrane proteins were determined by Western blots (Fig. 2D – lower panel). The concentrations of D1 and D2 proteins increased with leaf expansion and were initially lower in ffc than in wt. However, they reached near equivalent levels in fully expanded leaves, in contrast to LHC proteins. This is in agreement with earlier observations for whole seedlings grown of agar plates in continuous light (Pilgrim et al., 1998; Amin et al., 1999).

**Comparative proteome analysis of developing seedlings of ffc and wt using iTRAQ**

To further determine the consequences of the loss of cpSRP54 during early stages of leaf development, total cellular leaf proteomes of ffc and wt were compared using iTRAQ based protein quantification. To account for developmental delays that may mask direct effects of loss of cpSRP54, we compared young ffc seedlings with an average seven true leaves (stage 1.07) to wt seedling of the same age (21 days; stage 1.11) and wt seedlings of the same developmental stage (stage 1.07; 17 days old) (Fig. 3A). Plants these developmental stages are shown in Fig. 3B.

Total cellular leaf proteomes were extracted in the presence of SDS followed by removal of lipids and detergents in an optimized protocol for recovery of integral membrane proteins, and they were subsequently in-solution digested with trypsin. 30 μg of each digested proteome was labeled with the different iTRAQ reagents containing the 114, 115, 116 or 117 Da reporter (see Suppl. Fig.2). The labeled peptides were then mixed and separated using off-line strong cation exchange (SCX) chromatography (see Suppl. Fig. 2). Eight fractions from each SCX run were analyzed using on-line reverse phase nano-liquid chromatography electrospray tandem mass spectrometry (RP nanoLC-ESI-MS/MS). The three seedling stages (wt1.07, wt1.11 and ffc1.07)
were directly compared within each labeling experiment and a label switch was included to remove a possible labeling bias. These experiments were carried out with two independent biological replicates. The structure of this isobaric tagging reagent, the labeling scheme, labeling principle and quantification are explained in Suppl. Fig. 2.

123 proteins were identified unambiguously with one or more unique peptides (Suppl. Table 1A), using an established bioinformatics pipeline that included a decoy database search and a false positive peptide identification rate of less than 1% (Zybailov et al., 2008). In addition, 39 proteins were identified as part of small gene families, in particular in the LHC family and ribosomal proteins (Suppl. Table 1A). Relevant extracted information from the mass spectrometry analysis is shown in Suppl. Table 1A and can also be found in the Plant Proteomics Database, PPDB (http://ppdb.tc.cornell.edu/) (see Methods section). About 60% of the identified proteins are localized in the chloroplast, using the curated subcellular localization as described in (Zybailov et al., 2008). 19% of the identified proteins have at least one confirmed TMD, which is close to the predicted 22% for the whole Arabidopsis proteome (Sun et al., 2004).

The ffc/wt ratios of 97 proteins (or small families of proteins) were quantified in both biological replicates (Table 1); additional proteins quantified in only one replicate are not further discussed. Details of the quantifications (e.g. individual ffc/wt ratios for each peptide sequence) can be found in Supplemental Table 2. The average coefficient of variation (CV) for the ffc/wt ratio between the biological replicates was 15%. The quantified proteome covered mostly chloroplast stromal and thylakoid proteins (30% and 39%, respectively). Quantified proteins outside the chloroplast were enriched for peroxisomal and mitochondrial proteins involved in photorespiration, as well as cytosolic proteins involved in protein synthesis (Table 1). The ffc/wt ratios that are below 0.85 or above 1.15 and are also two standard deviations (stds) away from unity, are marked in bold (Table 2). This corresponds to a p value of <0.05, assuming normal distribution.

Differences between the two developmental stages in wild-type plants determined by iTRAQ
When comparing the two wt seedling developmental stages, it is notable that levels of major LHCl and II antennae proteins in the thylakoid proteome increase upto 50% during these four days of plant growth, reflecting the build-up of the photosynthetic apparatus during leaf development. This also underlines the importance of accounting for a developmental delay when
addressing the role of cpSRP54 and biogenesis of the LHC family. Interestingly, accumulation of the subunits of PSI and PSII core complexes and the thylakoid ATP-synthase complex increased on average only by 9%, 5% and 5%, respectively, indicating that assembly of the peripheral antennae complexes trails the accumulation of the PSI and II cores, as well as ATP synthase. Levels of 12 quantified Calvin cycle enzymes, and most other quantified functions inside and outside of the chloroplast did not change between stages 1.07 to 1.14 in wt seedlings (Table 1). We note that the ffc/wt ratios within these complexes and pathways were highly consistent, providing additional testimony for the accuracy and significance of the quantitative analysis.

**Differential protein accumulation between ffc and wt seedlings determined by iTRAQ**

*LHCI and LHCII proteins and PsbS.* In total, there are 22 LHC proteins in the antennae of PSI and PSII and they are arranged in six LHCII and five LHI subfamilies (Fig. 4). They contain a partially conserved ‘L18’ hydrophyllic domain located between TMD 2 and 3 (Fig 4); this L18 domain was shown to be essential for interaction with cpSRP43 and formation of the ‘transit complex’ (DeLille et al., 2000; Tu et al., 2000). We detected and quantified nine out of the 11 LHC subfamilies; their accumulation levels in ffc were between 70% and 100% of wt 1.07 levels. When comparing LHC levels in ffc 1.07 to wt 1.11, these accumulation levels were further reduced as a consequence of the developmental delay (Table 1). Compared to other LHC members, the LHCII-1 family was most reduced, while CP26 (LHCII-6) was unaffected. The abundant PsbS protein with pigment binding domains and four TMDs (rather than three in the LHC family) accumulated at 40% higher levels in ffc than in wt, in agreement with observations that PsbS does not require the SRP pathway *in vivo* (Tzvetkova-Chevolleau et al., 2007) and can stably accumulate in the thylakoid, independent of PSII (Niyogi et al., 2005).

*Photosynthetic thylakoid complexes* The four major complexes, namely PSII, PSI, the ATP-synthase and the Cytb6f complex, contain several chloroplast-encoded integral membrane proteins that are potential substrates of the cpSRP54 dependent co-translational insertion pathway. The accumulation levels of these chloroplast-encoded proteins could be directly affected by the lack of cpSRP54 in the ffc mutant. The nuclear-encoded subunits in these complexes are not known to require the cpSRP pathway.

Within the PSII core complex, 11 proteins were quantified (Table 1), including five chloroplast-encoded proteins (D1, D2, CP43, CP47 and cytb559 alpha), the nuclear-encoded
integral membrane core protein PsbR and the three nuclear-encoded proteins of the water splitting complex (OEC16, OEC23 and OEC33 and some of their homologues). The average accumulation of PSII core proteins in \textit{ffc} was 8\% reduced when compared to wt 1.07 and 19\% reduced when compared to wt 1.11. The Sec dependent OEC33 and the TAT dependent OEC23 were unaffected in \textit{ffc}, while the two TAT dependent OEC16 homologues showed a strong differential response, suggesting that they have complementary functions (Table 1). Five PSI core subunits were quantified and include the major PSI core subunits PsaA,B (chloroplast-encoded), PsaD, PsaF and PsaG (nuclear-encoded). An average reduction of 54\% for these PSI core subunits was observed when comparing \textit{ffc} to wt 1.07 which increased to 65\% when comparing \textit{ffc} to wt 1.11. When comparing the changes in accumulation for the core subunits of these two photosystems, it is clear that the PSI/PSII ratio is lower in \textit{ffc} as compared to wt. The PSI/PSII ratio (calculated from the average \textit{ffc}/wt ratio of the core subunits) does not change during the four days of development in wt (1.07/1.11 = 0.98), but is only about 70\% of the wt ratio in \textit{ffc} seedlings (0.70 for \textit{ffc}/wt1.07 and 0.67 for \textit{ffc}/wt1.11).

Three chloroplast-encoded (CF1\text{\textalpha,\textbeta,\textepsilon}) and two nuclear-encoded peripheral (CF1\text{\textdelta,\textgamma}) ATP synthase subunits, as well as one nuclear-encoded integral ATP synthase subunit (CF0-II) were quantified. The peripheral subunits of this complex were reduced by 15\% in \textit{ffc} as compared to wt 1.07 and 22\% compared to wt 1.11, while CF0-II was unaffected in \textit{ffc}. \textit{In vitro} studies suggested that the CF0-II protein inserts ‘spontaneously’ into the membrane (Michl et al., 1994) which is consistent with our observations.

\textit{Chloroplast protein synthesis and homeostasis.} Stromal chaperones (Cpn60, Cpn21, Hsp70) involved in protein folding and maturation, as well as a highly abundant protein isomerase without known function (ROC4), increased 40-89\% in \textit{ffc} as compared to wt 1.07 (Table 1). The increase was slightly higher (from 46-107\%) when compared to wt 1.11. ClpC1,2 levels increased 67\% in the \textit{ffc} mutant, both compared to wt1.07 and wt1.11. ClpC1,2 are members of the Hsp100 family and in particular ClpC1 has been shown to be involved in protein import, \textit{e.g.} (Kovacheva et al., 2007). ClpC1,2 are likely also involved in the delivery of proteins for degradation to the stromal ClpPR protease complex (Adam et al., 2006). Three abundant RNA binding proteins (CP29A, CP29B, CP31) involved in mRNA stability and the elongation factor and chaperone EF-Tu1 (Rao et al., 2004), accumulated to 50\% higher levels in \textit{ffc}. The
thylakoid membrane proteases FtsH1,5 accumulated to 69% higher levels in ffc compared to wt, suggesting an increased need for degradation within the thylakoid membrane.

**Calvin cycle, photorespiration and nitrogen assimilation.** The small and large subunits of Rubisco accumulated at slightly lower levels in ffc (Table 1). In contrast, 9 other Calvin Cycle enzymes accumulated on average at 38% (±13%) higher levels in ffc compared to wt 1.07 (and 45 ±11% compared to wt 1.11). Eight peroxisomal and mitochondrial proteins involved in various aspects of photorespiration were quantified. Glycolate oxidases 1 and 2, the first respiratory enzymes in the peroxisomes increased by 39% in ffc as compared to wt 1.07 (and 86% compared to wt 1.11). Consistently, catalases 2 and 3, involved in detoxification of hydrogen peroxide released by glycolate oxidases, increased 45% in ffc. Three mitochondrial photorespiratory enzymes were quantified. The P protein of the glycine decarboxylase complex increased by 61±7% in ffc when compared to wt 1.07 (and 118±19% when compared to wt 1.11), while the T subunit was unaffected. The enzyme immediately downstream of glycine decarboxylase, glycine-serine hydroxymethyltransferase (SHM1), increased by 61±7% in ffc when compared to wt 1.07 (118±19% compared to wt 1.11). Chloroplast stromal glutamate synthase (GS2), involved in the photorespiratory cycle as well as nitrogen assimilation, was not significantly affected in ffc.

**Cytosolic functions.** Nine cytosolic proteins involved in protein synthesis and folding were quantified (Table 1). However, several of peptides used for these quantifications matched to multiple members of the respective protein families, reducing the value of the quantification. Nevertheless, it appears that cytosolic protein homeostasis was not or only slightly affected (ffc/wt = 1.27±0.13). Three enzymes of the cytosolic S-Adenosylmethionine (SAM) cycle were quantified, but did not show any consistent change in ffc.

**Overview of comparative analysis of the ffc and wt chloroplast proteome from mature leaf rosettes**

To compare the mature chloroplast proteomes of wt and ffc, chloroplasts were isolated from fully grown rosettes prior to bolting from 40 day old wt plants and 47 day old ffc plants, both at growth stage 3.90, as defined by (Boyes et al., 2001) (see Fig. 3A,B). The isolated chloroplasts were fractionated into soluble stroma and thylakoid membranes. The isolated thylakoid membranes were stripped of luminal and peripheral proteins and the remaining membrane
proteomes of ffc and wt were compared using iTRAQ labeling and quantification by MS/MS analysis (See Suppl. Fig. 2 for the experimental design). The stromal proteomes of wt and ffc were compared using differential stable isotope labeling with cleavable isotope-coded affinity tags (ICAT) (Fig. 5) (Tao and Aebersold, 2003), as well as 2-DE gels using IPG strips in the first dimension and SDS-PAGE in the second dimension (Fig. 6). In addition, wt and ffc stroma were also analyzed by 2DE page using native gels in the first dimension (CN-PAGE) and SDS-PAGE in the second dimension (Suppl. Fig. 3).

**Comparative analysis of the ffc and wt chloroplast stromal proteomes from mature leaf rosettes by ICAT**

The stromal ICAT analysis was carried out in two biological replicates, and included a label switch between wt and ffc. An overview of the procedure is provided in figure 5 with an explanation provided in its legend. The two ICAT experiments identified 194 and 196 proteins respectively, totaling 271 proteins (all identifications with associated scores and sequences are available via PPDB; and see Suppl. Table 1B). Since on average only about 20% of tryptic peptides contain a cysteine, a significant proportion of the identified proteins were not quantified. Respectively 293 and 133 ICAT labeled peptide pairs were manually quantified in experiment 1 and 2 (Suppl. Table 3A,B). After removing peptides matched to proteins that did not pass the identification criteria, 267 and 126 peptides (experiments 1 and 2, respectively) were matched to 83 identified proteins (Suppl. Table 3C). We did not detect unlabeled cysteine containing peptides (even after searching for acrylamide adducts to cysteins – ‘propionamide C’ modification), indicating that the ICAT labeling was saturating. This was expected as the ICAT reagent is an efficient alkylating reagent that was added in large excess. 46 proteins were quantified in both biological replicates (Table 2), with an average CV for ffc/wt protein ratios of 14%. A few proteins were not quantified individually, but as small clusters of closely related family members (Table 2).

We focus on the 46 proteins that were quantified in both experiments and passed the strict identification filters (Table 2). These proteins include members of the major biosynthetic pathways (Calvin cycle, pentose phosphate pathway, minor carbohydrates, starch, amino acids, chlorophyll, sulfur/cysteine metabolism), as well as protein synthesis and folding (CPN60, cpHSP70, ROC4) and protein degradation (ClpC, Zn Metalloprotease AtZnMP). While most of
the ffc/wt protein ratios were close to one, levels of 12 quantified Calvin cycle enzymes were systematically reduced in ffc (average ffc/wt = 0.78±0.10), which is consistent with the reduced LHC cross section and decrease in growth rates (see section below on the thylakoid). Stromal ascorbate peroxidase (At4g08390) was significantly increased by 56%, suggesting increased level of H$_2$O$_2$ and consistent with two-fold up-regulation of CuZn superoxide dismutase (At2g23190) as shown by 2DE gel analysis (see next section). It was also clear that there was neither an increased demand for protein folding, nor for stromal protein degradation since the major chaperones systems Cpn60 and HSP70, as well as ClpC1,2 and the abundant stromal metalloprotease AtZnMP, accumulated at wt levels (Table 2). We finally note that three ClpPR subunits of the major stromal Clp protease complex (quantified only in one of the biological replicates) were also at wt levels (Suppl. Table 3C).

**Comparative Analysis of chloroplast stroma from mature leaf rosettes by 2D-IEF-SDS-PAGE**

The CN-PAGE analysis of ffc and wt stroma did not show any obvious differences (Suppl. Fig. 3). The Rubisco holocomplex of ~550 kDa represents 58% of the stromal protein mass in isolated chloroplast from fully developed *Arabidopsis* leaf rosettes (Peltier et al., 2006). This strongly reduces the resolving power of 2DE gels using IEF as the first dimension. Since our previous and current CN-PAGE gel analysis showed that most abundant stromal proteins are found in monomers or complexes below 550 kDa (Peltier et al., 2006), we fractionated stroma from wt and ffc by sucrose density centrifugation and collected proteins sedimenting above the Rubisco complex as two fractions (fractions 1 and 2). These 2 fractions were each focused in the 1$^\text{st}$ dimension on IPG strips and then separated by SDS-PAGE in the 2$^\text{nd}$ dimension (Fig 6). The experiment was carried out in duplicate using independent chloroplast preparations, and including a technical replicate for fraction 1, resulting in 12 2DE gels. Protein spots were then matched across the 12 gels and quantified using image analysis software. The most abundant ~150 spots were analyzed by peptide mass finger printing using Matrix-Assisted Laser Desorption Ionization Time-of Flight (MALDI-TOF) MS to ensure correct spot matching (data not shown) and to identify proteins in up-and down-regulated spots.

We were able to match and quantify 197 spots across fraction 1 and 109 spots in fraction 2, while 101 spots were matched and quantified across both fractions. Most spots did not show significant changes, consistent with the ICAT analysis of the stroma (presented above) and
showing that proteome homeostasis returned to steady state in fully developed leaves. However, we did find significant (>2 fold stdev) changes for CuZn superoxide dismutase (At2g28190) and an oxidoreductase-like protein of unknown function (At1g23740) (Fig 7). Both proteins likely relate to ROS defense and/or redox regulation (see further in DISCUSSION).

Comparative analysis of the ffc and wt thylakoid proteomes from mature leaf rosettes using iTRAQ

The two independent stripped thylakoid preparations of wt and ffc were digested with trypsin and labeled with iTRAQ reagents (114,115,116,117) and a label switch was included for each independent preparation (see Suppl. Fig. 2). The two sets were fractionated by off-line SCX chromatography and fractions were analyzed by nanoLC-ESI-MS/MS similar to the seedling samples, discussed above. This unambiguously identified 65 proteins with one or more uniquely matched peptides, and an additional nine proteins were identified as part of small protein families (Suppl. Table 1C and see PPDB). We quantified 42 proteins in both biological replicates and only those are considered (Table 3).

Accumulation levels of LHCI and major LHCII were reduced between 20 and 30% in ffc, while accumulation of minor LHC proteins CP24, CP26 and CP29.1 were unaffected or even slightly increased. However, CP29.2 decreased by 40%. Accumulation levels of ten quantified PSII core proteins in ffc increased on average by 12%, while the six quantified PSI core proteins decreased on average by 20%. Consequently, the PSI/PSII core ratio (determined by the average ffc/wt ratio for the core subunits) decreased by 30% in ffc as compared to wt in full grown plants. Accumulation levels of subunits of ATPsynthase and cyt b6f complex did not change. Interestingly, accumulation levels of thylakoid proteases FtsH2,8 doubled in ffc suggesting an increased need for degradation of thylakoid proteins, similar to the findings in young seedlings (Table 3).

Genetic interaction between cpSRP54 and ClpC1

A central component in chloroplast protein biogenesis is the AAA+ chaperone ClpC1 (At5g50920). ClpC1 predominantly accumulates in the chloroplast stroma, but has also been shown to interact with the inner envelope protein import complex (Nielsen et al., 1997). A null mutant in CLPC1 was isolated and has a virescent phenotype and reduced protein import rates
A null mutant in a closely related homologue expressed at lower levels, ClpC2 (At3g48870), does not have any visible phenotype (Kovacheva et al., 2005). ClpC is not only involved in the chloroplast import process, but also likely targets proteins to the ClpPR core complex for degradation (Adam et al., 2006; Sakamoto, 2006). It is conceivable that the functional association of ClpC with the Tic complex is to keep this major protein import pathway free from substrates that are trapped in the translocon. The comparative proteomics analysis of young \textit{ffc} and wt seedlings described above, as well as previous western blotting data (Pilgrim et al., 1998; Hutin et al., 2002), showed that ClpC1,2 levels are increased by 30-50\% in young \textit{ffc} plants.

Given the relevance of ClpC1 in both import and degradation, we isolated a ClpC1 null mutant, \textit{clpc1-1} and crossed it with \textit{ffc}. Double homozygous mutants were recovered in the F2 generation and RT-PCR confirmed the lack mRNA for both \textit{cpSRP54} and \textit{CLPC1} (Fig. 8). The double mutant was blocked in development after emergence of the cotyledons under autotrophic conditions, but could be partially rescued under heterotrophic conditions (1\% sucrose) (Fig. 8). Under these heterotrophic conditions, the double mutant developed and greened slowly and could eventually be transferred to soil. After about 9 months, the plants finally flowered and produced low amounts of viable seed. The strong phenotype confirms the significance of the iTRAQ based quantification in young seedlings and indicates that ClpC1 is of particular importance when intraplastid sorting is disturbed.

**DISCUSSION**

Protein sorting is a key process in development and function of eukaryotic and prokaryotic organisms; disruption of this process often leads to developmental and functional defects. Since protein sorting is so vital, redundancies are often built-in, while various rescue mechanisms are in place to either remove miss-sorted and aggregated proteins through proteolysis, or rescue them via chaperones (Bukau et al., 2006). Therefore, to study protein sorting pathways and the consequence of inactivation of specific sorting components, an integrative approach is needed that can monitor many of the protein homeostasis components as well as other cellular functions in parallel. Gel-based and, in particular, MS based techniques to determine quantitative differences between proteomes have greatly improved in recent years (Goshe and Smith, 2003; Domon and Aebersold, 2006; Bantscheff et al., 2007) and several of these comparative and
quantitative techniques have been used to study plants – reviewed in (Jorrin et al., 2007; Thelen and Peck, 2007). We present a comparative proteomics approach to address the intra-chloroplast sorting and homeostasis network and to determine the cellular response outside of the chloroplast.

**Quantitative proteomics methodologies for analysis of chloroplast and other mutants affecting leaf development**

We used MS-based (iTRAQ and ICAT) and 2DE gel-based quantification techniques to study the impact of cpSRP54 deletion in both young seedlings and mature rosettes (see Fig. 3 for overview). To analyze young seedlings, we optimized a procedure for extraction of total cellular proteins, including membrane and soluble proteins, avoiding any gel-based steps. Proteins were extracted with SDS and could be efficiently digested after solubilization in DMSO, similar as we previously demonstrated for the proteome of plastoglobules (Ytterberg et al., 2006). Importantly, membrane proteins were well represented using this method. This gel-free digestion procedure is also particularly attractive when using the iTRAQ peptide labeling technique. Since the ffè mutant is affected in leaf and chloroplast development (Figs. 1, 2), we included a comparison based on similar leaf age, as well as similar leaf growth stage. This was important, as even a difference of four days in plant age significantly affected thylakoid composition in these young seedlings (but not other compartments) (Table 1). The seedling analysis identified and quantified mostly chloroplast proteins (60%), making this an attractive general method for the analysis of chloroplast mutants, especially if these mutants have strong phenotypes from which it will be difficult to isolate significant amounts of intact chloroplasts.

For a more detailed analysis of the purified chloroplast stromal proteome isolated from fully developed leaf rosettes, we used ICAT (Table 2 and Fig. 5), as well as 2DE gel analysis (Figs. 6, 7). To overcome the loss of resolution by the presence of high amounts of Rubisco, we used a simple and reproducible sucrose gradient fractionation step prior to 2DE gel analysis. The ICAT and 2DE gel analysis was complementary and consistently showed that protein homeostasis reached steady state without folding or degradation ‘stress’. The CVs of quantified protein ratios across biological replicates using the iTRAQ and ICAT methods were very similar (14-15%). A clear disadvantage of the ICAT method was that a significant number of identified proteins could not be quantified due the dependence on the presence of cysteines.
The post-translational SRP sorting machinery but not the SecY/E translocon is down-regulated upon development

Protein import and sorting rates are particularly high during early leaf and chloroplast development to sustain rapid cell division and expansion. As the leaf matures, import and sorting slows down and the leaf proteome reaches steady-state. We found that protein accumulation levels of cpSRP54, cpSRP43, cpFtsY and ALB3, but not SecY/E, decreased during leaf development when based on total protein concentration (Fig. 2), and even more if chlorophyll concentration would be used for normalization. Down-regulation of these SRP targeting components with progressive leaf development has not been shown before, but is consistent with a decreasing need for this biogenesis machinery as more of the thylakoid membrane system is developed. When seedlings of the same developmental stage were compared, levels of each of these sorting components were similar. The relative stable accumulation levels of the SecYE translocon are in contrast to the down-regulation of the SRP pathway components. This suggests that the Sec translocon has a sustained role in chloroplast proteome homeostasis, possibly to accommodate the D1 synthesis and repair cycle of PSII (Aro et al., 2005).

Substrates for cpSRP54 in the LHC family and significance of the L18 domain

We quantified a number of LHC proteins in both young seedlings and developed rosettes. All LCH proteins were reduced except for CP26 in young seedlings, while in fully developed rosettes all quantified LHCs except for CP24, CP26, CP29-1 were reduced. In particular, these minor LHCII proteins accumulated at higher levels in fully developed rosettes, which is consistent with Western blot analysis (Amin et al., 1999). This also supports the idea that sorting can proceed using only cpSRP43 when cpSRP54 (normally interacting with LHC substrate via hydrophobic TMDs) is not present, in agreement with previous observations (Amin et al., 1999)(Hutin et al., 2002)(Tzvetkova-Chevolleau et al., 2007).

While it has been established that the L18 domain is required for cpSRP43 interaction - reviewed in (Schunemann, 2004)-, it is not clear which residues within the L18 domain are important. From the alignment of the L18 domains of all Arabidopsis LHC proteins, we observed that the L18 domain is generally conserved, but that a number of LHCs, in particular the CP29 family, CP26 and LHCI-1.1 have one or more basic residues upstream or downstream of the
central YPGG domain; this changes the overall properties of this cpSRP43 interaction domain (Fig. 4) One could argue that in absence of the LHC interaction with cpSRP54, the properties of the L18 domain determine how successful LHC can still be targeted to the thylakoid membrane via cpSRP43 alone, thus providing a explanation of the behavior of most minor LHIIs. However, CP29.2 and LHCI-1 did not follow this pattern, indicating that features other than the L18 domain in the LHCs contribute to the efficiency to bypass cpSRP54.

**Consequences for c-encoded proteins and decreased PSI/PSII core ratio**

The most pronounced and persistent effect on the thylakoid proteome was the reduction in accumulation of PSI core subunits and consequently, a decrease in PSI/PSII ratio. The strong effects on these PSI subunits is consistent with immunoblot data for PsaA,B in previous in vivo studies (Pilgrim et al., 1998; Amin et al., 1999). These two chloroplast-encoded reaction center proteins are very hydrophobic (GRAVY index 0.25 and 0.12, respectively) with 9-11 TMDs – and their biosynthesis and membrane assembly is likely sensitive to aggregation. The reduced accumulation of these two chloroplast-encoded proteins is mostly likely a consequence of their co-translational dependence on cpSRP54, although direct evidence is lacking. In contrast, the decline in accumulation of the nuclear-encoded PSI subunits may not be directly related to loss of sorting efficiency, but could rather be a consequence of reduced accumulation of PsaA,B and subsequent degradation by the thylakoid membrane proteases. Indeed, we found that thylakoid FtsH1,5 proteins were up-regulated in both young and fully developed leaf rosettes (Tables 1,3). The lower steady state PSI/PSII ratio could also be a consequence of the reduced cross section of the PSII antennae. In addition, there appears to be changes in the relative contributions of the minor LHC members (CP42, CP26 and CP29) which may affect efficiency of the LHCII antennae, thus affecting the PSI/PSII balance.

**The ffc phenotype changes during progressive leaf and chloroplast development**

The proteomics analysis of the ffc mutant showed that the protein homeostasis machinery and the Calvin cycle enzymes responded differently in young as compared to mature leaves (Tables 1,2). In particular, cpSRP54 deletion led in young leaves to up-regulation of thylakoid proteases and stromal chaperones, including ClpC, and various RNA binding proteins. The RNA binding proteins have been shown to be vital for the stability of chloroplast mRNA and they have been
suggested to be global mediators of chloroplast RNA metabolism connecting transcription and translation in chloroplasts (Nakamura et al., 2004). In contrast, the stromal protein homeostasis machinery returned to wt levels in mature leaves, consistent with the developmental down-regulation of the SRP pathway. A differential response between young and mature leaves was also found in carbon metabolism, with an up-regulation of the Calvin cycle, the envelope triosephosphate translocator (Weber et al., 2005), and the photorespiratory pathway in peroxisomes and mitochondria. Since photorespiration exports reducing equivalents from the chloroplasts, this suggests a low ATP/NADPH production ratio by the photosynthetic light reaction. The up-regulation of most Calvin cycle enzymes is best explained by a partial uncoupling or insufficient coordination between expression of the Calvin cycle enzymes and thylakoid biogenesis during periods of rapid cell growth and division. In contrast, the Calvin cycle was down-regulated in mature leaves, likely to adjust to the reduced capacity of the light reaction, while ROS defense proteins (CuZnSOD, sAPX and the uncharacterized oxidoreductase-like protein) were up-regulated. This increase in ROS defense points to production of superoxide/hydrogen peroxide consistent with a low ATP/NADPH production ratio. This may well relate to the decreased PSI/PSII ration and consequently, a reduced cyclic electron flow capacity. These secondary effects of cpSRP54 deletion were otherwise confined to the chloroplast since cytosolic glycolysis, nitrogen transport and the SAM cycle proteins, involved in the biosynthesis of methionine and S-adenosylmethionine, did not change.

The double mutant \textit{ffc x clpc1} showed a much stronger phenotype than either single mutant (Fig. 8), which confirms the significance of up-regulation of ClpC chaperone in the young leaves. It has been shown that ClpC interacts in a reversible manner with the Tic translocon (in particular Toc110) (Nielsen et al., 1997; Kovacheva et al., 2007) and that loss of ClpC1 decreases import efficiency of various substrates (Constan et al., 2004; Kovacheva et al., 2005). ClpC likely assists the translocation of substrates by pulling the substrate out of the translocon in an ATP dependent manner. LHC sorting to the thylakoid is clearly less efficient and ClpC could help to keep this major protein import pathway free from substrates that are trapped in the translocon. The observed increases in CPN60, HSP70 support a role in LHC stabilization, possibly compensating for the lack of stabilization by cpSRP54.

\textbf{METHODS}
Generation and genotyping of single and double mutants, plant growth and treatments

The \textit{ffc} mutant previously characterized in (Amin et al., 1999) was originally isolated from an EMS mutagenesis screen and shown to have a \(~\text{10 kb}\) insertion in intron 8 of the gene encoding \textit{cpSRP54} (At5g03940.1). The \textit{clpc1} mutant is a T-DNA insertional mutant in At5g50920 in Col-0 ecotype obtained from the SALK collection (SALK_014058). The T-DNA is inserted in the fourth exon, at 1332 bp from the start codon, which was confirmed by DNA sequencing. The \textit{ffc} mutant was crossed to \textit{clpc1} and the F2 population was screened on soil in short days (10h light, 14h dark). No double homozygotes were found, but screening the F2 population on 1/2x MS plates containing 1\% sucrose in short days identified an \textit{ffc/clpc1} double mutant. \textit{ffc/clpc1} double mutant plants exhibit seedling lethality when grown only on MS media without sucrose in short days (10h light, 14h dark). mRNA levels in \textit{wt}, \textit{clpc1}, \textit{ffc} and \textit{ffc/clpc1} were determined from 24 day old plants grown in short days (10h day, 14h night) on ½x MS media containing 1\% sucrose by RT-PCR. Total RNA was isolated from \(~\text{100mg}\) tissue from these plants using Tri-Reagent (Molecular Research Center, Inc. Cincinnati, OH). cDNA was amplified from 2 \(\mu\mathrm{g}\) total leaf RNA with Superscript III (Invitrogen Corp., Carlsbad, CA). Primers for \textit{cpSRP54} (for. 5’-GAGGCTCTTCAATTTTCCAGCG-3’, rev. 5’-CAGGCTTGCTTGATGCTGACTC-3’) were used to amplify the entire coding sequence of 1663 bp. Primers for \textit{CLPCl} (for. 5’-ATGGCTATGGCCACAAGGGTGTTG-3’, rev. 5’-GATCTTCTGGGTACAGCTCACAATATTG-3’) were used to amplify a 1024bp segment at the N terminal portion of the gene.

For chloroplast isolation, stroma purification, thylakoid preparation, and total plant protein isolation, wild-type (Col. 0) and \textit{ffc} plants were grown on soil under a 10 hrs light/14 hrs dark period at 22/19 \(^\circ\)C at 280 \(\mu\text{mol}\) photons m\(^{-2}\)sec\(^{-2}\).

Preparation of whole leaf extracts from different leaf stages for immunoblot analysis

Individual leaf pairs at defined stages were collected and frozen in liquid nitrogen. Around 20-30 leaves were pooled for small leaves (2-5 mm), around 8-10 for medium sized leaves (5-10 mm) and 2-6 for big leaves (<10mm). Frozen leaves were ground with pestel and mortel in 500 \(\mu\text{L}\) medium containing 50 mM Tris-HCl (pH 6.8), 2 mM EDTA, and 50 \(\mu\text{g/ml}\) water soluble irreversible serine protease inhibitor pefablok ((4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride) (Biomol).
and subsequently filtered through nylon mesh (22 micron). Proteins were then either precipitated in 80% acetone (the supernatant was used to determine chlorophyll concentrations (Porra, 1989)) or thylakoid membranes were collected by centrifugation at 12k xg for 3 min. Protein pellets were solubilized in 50-100 μl of 5% SDS and 50 mM Tris-HCl (pH 8.3) and thylakoid membrane pellets were solubilized in 100μl 6M Urea, 5% SDS, 0.3 M sucrose and 50 mM Tris-HCl (pH8.3).

Before starting the Western blotting analysis of the leaf pairs, titrations were carried out to determine the linearity of the antibodies directed against cpSRP54, cpSRP43, cpFtsY, cpSecY and ALB3, using wt and ffc1-2 leaf material from young leaves (not shown). Based on these titrations, 50 μg of total leaf protein extract was loaded for analysis of cpSRP54, 20 μg for cpSRP43, and 30 μg for cpFtsY and ALB3. Thylakoid membranes corresponding to 100 μg total leaf protein extract were loaded for analysis of cpSecY and cpSecE. A fragment of ALB3 (aa 327-462) was overexpressed in E. coli, gel purified and injected into rabbits for production of polyclonal antiserum (AgriSera, Sweden) and the serum was used at 1:1000. Generation of cpSecE serum is described in (Froderberg et al., 2001). Rabbit polyclonal antisera raised against cpSecY (used at 1:2000), cpFtsY (used at 1:2000) and cpSRP54 (used at 1:1000) were kind gifts from Neil E. Hoffmann. The cpSRP43 sera (used at 1:3000) were kind gifts of Laurent Nussaume and Ralph Henry. Proteins were separated on 12 or 14% Laemmli SDS-PAGE gels containing 6M urea and blotted onto PVDF membranes. For cpSecE, proteins were separated on an 8-16% gradient Tricine SDS-PAGE gel containing 6 M urea and blotted onto a PVDF membrane. Bound primary antisera were detected with secondary antibodies conjugated to Horseradish peroxidase (Sigma) and detected by enhanced chemiluminescence.

**Isolation of total cellular proteome for seedling analysis by iTRAQ**
Proteins from total tissue were isolated from wt and ffc seedlings at growth stage 1.07 (wt and ffc at 17 and 21 days respectively) and for wt seedlings at growth stage 1.11 (wt at 21 days). Ten seedlings (leaf tissue only) of each developmental stage and genotype were ground in extraction buffer (6 M urea, 5% SDS, 0.3 M sucrose, 50 mM Tris-HCl 8.3, 5 mM tributylphosphine) in a mortar. The supernatant was filtered through a 45 μm miracloth and insoluble material was removed by centrifugation at 18k xg for 1 min. at 4°C. Each sample was precipitated in 80% 100% acetone at -20°C. The protein pellets were collected by centrifugation 18k xg for 5 min at
4°C followed by solubilization in 2% SDS and protein determination using the bicinchoninic acid method (Smith et al., 1985).

**Isolation of the stripped thylakoid proteome for analysis by iTRAQ**

Thylakoids were purified from fully developed leaves using a combination of differential- and gradient centrifugation steps as described in (Friso et al., 2004). Soluble and peripheral proteins were removed by stripping the thylakoid membrane as follows: thylakoid membranes were resuspended at 10 mg chlorophyll ml⁻¹ in ice-cold 10 mM Hepes-KOH (pH 8.0) buffer containing a cocktail of protease inhibitors as listed in (Peltier et al., 2002). The suspension was diluted 20 times with ice-cold solutions of 2 M KBr and KNO₃ to a final concentration of 1 M. The suspension was stirred slowly for 30 min on ice with 10s sonication steps every 5 min. Membranes were collected by centrifugation at 150k xg for 25 min at 4°C, washed with 10 mM Hepes-KOH (pH 8.0) buffer with sonication to remove the remaining salts, followed by centrifugation at 150k xg for 15 min to collect the stripped membranes. The membranes were then solubilized with 50 mM Tris-HCl (pH 8.0), 3 M Urea, and 2% SDS.

**Removal of SDS, in-solution digestion and iTRAQ labeling**

30 μg protein of each sample was incubated over night at -20°C with 450 μl 100% acetone followed by centrifugation (18k xg, 4°C, 15 min), washing the pellet with 80% acetone, 10% methanol, 0.2% acetic acid, 9.8% water in acetone, followed by 30 min incubation at -20°C, followed by a final centrifugation (18k xg, 4°C, 15 min) to collect the protein. The proteins were solubilized in 20 μl 100% DMSO, followed by dilution with digestion buffer to a final concentration of 50 mM ammonium bicarbonate and 30% DMSO. After adding trypsin to a protein:trypsin ratio of 20:1 (and a final volume of 67μl), the samples were incubated on a shaker over night at 37°C. After digestion, the DMSO was removed by evaporation in a speed vac and the peptides dissolved in 100 μl 5% formic acid (FA). 13 μg (43.3 μl) from each sample were aliquoted into two tubes and dried down. 100 μl water were added to each tube and evaporated to remove residual ammonium bicarbonate. 30 μl 0.5 M triethyl ammonium bicarbonate was added to each tube and vortexed. Each peptide sample was mixed with a whole tube of labeling iTRAQ reagent (114, 115, 116 or 117; Applied Biosystems) and incubated on a shaker at RT for 1 hour. The samples to be compared were pooled and 50 μl 5% FA per sample was added per label to set
the pH. Finally the solutions were diluted 10 times the reaction volume with 25% acetonitrile (ACN) and 1% FA (final concentration < 0.05 M triethylammonia bicarbonate).

**Strong cationic exchange chromatography of iTRAQ labeled samples**

The iTRAQ labeled peptides were separated using SCX chromatography on a PolySULFOETHYL A™ column (200 x 2.1 mm, 5 µm, 300 Å) from PolyLC Inc connected to an Agilent 1100 HPLC. Solution A was 25% ACN and solution B was 25% ACN, 0.5 M ammonium formate (pH 3.0 - set with FA). 100 µl of each sample was injected five times (3%B 0-3 minutes) before eluting the peptides off the column at a flow rate of 200 µl/min as follows: 3% B 0-10 min, 3-10% at 10-15 min, 10-60% at 15-20 min, 60-100% 20-25 min, 100% 25-35 min, 100-3% 35-37 min, 3% 37-45 min). The fractions were collected in microtiter plates (start at 2 min, end at 45; 1 min/fraction; ~3 min dead volume). The fractions were lyophilized and resuspended with 5% FA.

**Mass spectrometry analysis of iTRAQ samples**

For identification and quantification the eight SCX fractions containing the majority of the peptides (fraction 23-30) were analyzed data dependent on-line tandem mass spectrometry using a CapLC-ESI-MS/MS (Q-TOF1; Waters), similar as described in (Zybailov et al., 2008). Proteins were identified by searching the MS data against ATH1v6 database concatenated with a decoy where all sequences were in reversed orientation using Mascot with a significance threshold of 0.01. The maximum mass error tolerance for precursor and products ions was set at respectively 1.2 and 0.8 Da. Search criteria were as follows: full tryptic peptides only, variable methionine oxidation, fixed iTRAQ modification for N-termini and lysine residues and variable iTRAQ modification for tyrosine residues. In addition, only MS/MS spectra with ion score of 20 or higher were considered for identification, but lower ion scores were allowed for quantification.

**Calculation of protein ratios and quality control of iTRAQ data**

Mascot distiller was used to calculate peak areas and extract m/z of the precursor ions, area and retention time. The peak areas of the reporter ions were extracted and then corrected for label purity with parameters supplied from Applied Biosystems using program written by Dr. Qi Sun.
(Theory Center, Cornell University). The quantified reporter ion information for each fragmented precursor ion was coupled to the queries matched to protein accessions; quantifications for queries that were not used for identification were removed. When queries where used to identify more than one peptide, only the best match (i.e. the best interpretation of the spectrum) were kept, unless it seem that more than one peptide was fragmented at the same time. In the latter case the spectrum was used for the normalization, but discarded for the statistical analysis of the proteins, since the reporter ions would originate from two different sources. After normalization, the peptides were grouped according to peptide sequence and the area of reporter ions originating from the same peptide sequence were added together (i.e. repeated fragmentation of the same ion, or fragmentation of peptides with different modification such as oxidized methionines) to prevent one peptide sequence from biasing the protein average. The peptides were then grouped according to the proteins they matched to. The sequences of the proteins that had shared peptides were aligned using Multi-align (Corpet, 1988) and the peptide sequence matched to the alignment to clarify peptide/protein relationship. Unique and shared peptides were kept separate to not compromise the data and averages and standard deviations for the individual biological replicates were calculated using the ratios from the labeling experiments. Outliers were removed conservatively using the standard deviation.

2D-PAGE analysis of chloroplast stroma of ffc and wt plants

Isolation of chloroplasts and extraction of stromal proteins was carried out as described in (van Wijk et al., 2007). 2 mg of purified total concentrated stroma from both wt (Col-0) and ffc chloroplasts was loaded onto a 2 ml 10-30% continuous sucrose gradient (in 10 mM Hepes-KOH pH 8.0, 5 mM MgCl₂, protease inhibitors). Gradients were spun at 4C for 3h at 55K rpm in Ultracentrifuge (Beckman-Coulter) and manually fractionated at 4C into 4 fractions. 150 µg protein of each fraction 1 and fraction 2 was precipitated in 80% acetone, resuspended in rehydration buffer (final concentration) and rehydrated onto 11 cm pI 4-7 IPG strips (Amersham Pharmacia). Strips were focused in the 1st dimension, reduced, alkylated, and run in the 2nd dimension on 8-16% Tris-HCl Criterion gels (BioRad). Gels were fixed in 10% Methanol and 7% Acetic Acid for 1h and stained with Sypro Ruby O/N. Gels were then destained in fix solution, rinsed with ddH₂O for 15 minutes and images were acquired with a Fluor-S unit (BioRad). Spot quantification was performed using Phoretix 2D software. Final spot volumes are
normalized to total gel volume for each gel. Stained gel spots were excised manually or using a ProPic robot (Genomic Solutions; Ann Arbor). The spots were washed, digested with modified trypsin (Promega) and extracted using a ProGest robot (Genomic Solutions; Ann Arbor). The extracted peptides were dried and re-suspended in 15 μl 5% FA. Protein identification was performed by peptide mass finger printing using Matrix-Assisted-Laser-Desorption Ionization Time-of-Flight MS in reflectron mode (MALDI-TOF MS; Voyager DE-STR from Perseptive Biosystems). Peak lists (mgf files) from the MALDI data were generated using MoverZ software m/z (Freeware edition; Proteometrics, Inc.) using a minimum signal to noise ratio of 3.0 and peak resolution of 5000. The peak lists were searched against Athv6 using Mascot v2.2 with a maximum p value of 0.05. Criteria for positive identification by MALDI-TOF MS peptide mass finger printing include five or more matching peptides with a narrow error distribution (clustering of errors) within 25 ppm and at least 15% sequence coverage. Only peptides without missed cleavages (by trypsin) were considered, with methionine oxidation as variable modification and carbamido-methylation as fixed modification. In exceptional cases (i.e. proteins less than 20 kDa and matching gel coordinates) four matching peptides were considered as positive identification.

**Stable isotope labeling using ICAT and Mass spectrometry analysis**

200 μg of purified stromal proteins from wt and from ffc were denatured and reduced using 50 mM TCEP-HCl. All cysteine residues were labeled with the light (containing only 12C stable isotopes) or heavy (13C) ICAT reagent according to the manufacturer’s instructions (Applied Biosystem). Labeling reactions were performed separately by incubation at 37ºC for 2 hours. After labeling, the samples were combined and proteins separated by 1-dimensional SDS-PAGE (12% acrylamide), followed by Coomassie Brilliant Blue (R-250) staining. The gel lane containing the ICAT labeled samples was completely cut into 12 slices. Each slice was cut into 1mm³ cubes, washed and digested with modified trypsin (Promega) and the resulting peptides were eluted principally as in (Shevchenko et al., 1996). After vacuum concentration, 30 μl of 25% ACN, 5 mM KH2PO4, 350 mM KCl (pH2.7) and 500 μl of affinity-load buffer (pH 7.2) were added to each sample as described in (Li et al., 2003). The biotin-tagged peptides were purified on avidin columns as instructed by the manufacturer. Protein digests were qualitatively analyzed on a MALDI-TOF mass spectrometer (Voyager DE-STR; Applied Biosystems) to
confirm total digestion and/or labeling. (Labeled) peptides were then loaded on a guard column (LC Packings; MGU-30-C18PM), followed by separation on a PepMap C18 reverse-phase nano column (LC Packings nan75-15-03-C18PM), using 90-min gradients with 95% water, 5% ACN, 0.1% FA (solvent A), and 95% ACN, 5% water, 0.1% FA (solvent B) at a flow rate of 200 nl/min. The labeled peptide mixtures were analyzed by LC-ESI-MS/MS (Q-TOF). Each ICAT-labeled sample was run in duplicate under the same chromatographic settings. For protein quantification the sample were first analyzed in MS mode and the area of the peptides were calculated using Masslynx 4.0 SP1. The second run was set up for data dependent MS/MS acquisition for protein identification. The flow-through from the 12 avidin column purifications (containing non-labeled peptides, i.e. peptides without cysteines) were also collected and analyzed by MS/MS (peptides not usable for quantification but valuable for protein identification) after off-line desalting on C18 microcolums (Gobom et al., 1999). The experiment was carried out in duplo with independent chloroplast preparations and ‘label switch’ to avoid a possible bias due to the ICAT label. All quantified peptides and details are listed in Supplemental Table 3. Criteria for protein identification are similar as for the iTRAQ labeled peptides, except that a variable ICAT modification was included (instead of variable iTRAQ modification).

The Plant Proteomics database, PPDB

Mass spectrometry based information of all identified proteins listed in Supplemental Table 1A,B,C was extracted from the Mascot search pages and filtered for significance (e.g. minimum ion scores, etc) and ambiguities and shared peptides as described in (Zybailov et al., 2008). This information includes Mowse score, number of matching peptides, number of matched MS/MS spectra (queries), number of unique queries, highest peptide score, highest peptide error (in ppm), lowest absolute error (ppm), sequence coverage, tryptic peptide sequences). This information is available either by using the search function ‘Proteome Experiments’ and selecting the desired output parameters. This search can be restricted to specific experiments. Alternatively, information for specific accessions (either individually or a group) can be extracted using the search function ‘Accessions’ and, if desired, this search can be limited to specific experiments. Finally, information for a particular accession can also be found on each ‘protein report page’. The MapMan Bin system (Thimm et al., 2004) is used for functional assignment and all assignments for identified proteins were verified manually. Experiments


numbers for data presented in the current study are as follows: For iTRAQ analysis of total seedling extracts: experiments 395, 396; For iTRAQ analysis of thylakoids from fully developed leaf rosettes – experiments 393, 394; For iCAT analysis of stroma of fully developed leaf rosettes – experiments 194/195 and 249/250.

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FIGURE LEGENDS

Figure 1. Growth and development of A. thaliana (col) wt and ffc plants grown on soil under four different photon flux densities. A, Photos of 33 day old plants grown under four different photon flux densities. All bars (in white) correspond to 20 mm. B, Diameter of rosettes of 33 days old wt (white) and ffc plants (grey) grown under the different light intensities. Dim light: 70 μmol m^{-2} s^{-1}, low light: 165 μmol photons m^{-2} s^{-1}, medium light: 320 μmol photons m^{-2} s^{-1}, and high light: 600 μmol photons m^{-2} s^{-1}.

Figure 2. Analysis of chloroplast targeting components and thylakoid proteins in wt and ffc1-2 during leaf development. Individual leaf pairs and plant developmental stages are indicated. Accumulation of ALB3, cpFtsY, cpSRP54, cpSRP43 (A) and cpSecY (B) and cpSecE (C) in individual leaf pairs during plant development. Protein loads were 30 μg for ALB3, cpFtsY, 50 μg for cpSRP54, 20 μg for cpSRP43 and the membrane fraction from 100 μg of total proteins for cpSecY. D, Expression analysis of the major LHCPII, D1 and D2 protein, during leaf development in wild-type and ffc1-2. Accumulation of the LHCII family (between 25-27 kDa) was determined by 1-DE gels and silver staining in leaves 3&4 and 7&8 with progressive plant development. Accumulation of chloroplast-encoded thylakoid membrane proteins D1 and D2
during leaf development of leaf pairs 3&4 and 7&8 was determined by Western blot analysis. Total leaf extracts were loaded.

**Figure 3.** Mutant phenotype and developmental stage comparison. A, The developmental time line of *ffc* and wt plants. Plants were harvested at 17 days (wt) and 21 days (wt and *ffc*) for the comparison of total cellular proteomes and analyzed using the isobaric reagent iTRAQ followed by mass spectrometry analysis. Stripped thylakoid membranes and soluble stroma proteins were collected from chloroplasts isolated from mature plants (40 and 47 days). The soluble stromal proteomes were analyzed using the isotopic labeling reagent ICAT followed by mass spectrometry analysis and by 2DE gel analysis, while the thylakoid proteomes were compared using iTRAQ. B, Phenotype of the *ffc* mutant with delayed development, smaller stature and paler color. At full grown rosette stage, the difference between *ffc* and wt is much less pronounced.

**Figure 4.** Alignment of the L18 motif from pea Lhcb1 and all 22 *Arabidopsis* LHC proteins as well as PbsS. *ffc*/wt ratios for young seedlings (stage 1.07) and for fully developed rosette leaves (M) as determined by iTRAQ analysis are indicated. Y = young seedlings. M = mature plants. *This ratio has a very high CV.*

**Figure 5.** Schematic overview of the ICAT labeling experiments of wt and *ffc* chloroplast stroma. 200 μg of each wt and *ffc* denatured stromal proteins were labeled with the heavy or light ICAT tags. Mixed *ffc* and wt labeled proteomes were fractionated by 1D SDS-PAGE and, stained by Coomassie blue. The entire gel lane was cut into 12 gel slices and proteins were digested with trypsin. After peptide extraction, ICAT modified peptides were purified on an avidin column. After elution, the biotin tag was removed by acid-cleavage and the peptide mixture analyzed by RP-nanoLC-ESI-MS. The 12 samples were first analyzed in MS mode to acquire an ion chromatogram for quantification and subsequently also in MS/MS mode for peptide identification. Peptides that lack cysteines can not be labeled with the ICAT method and eluted with the flow-through from the avidin columns. These unlabelled peptides were collected and analyzed by MS/MS to obtain a better coverage of the stromal proteome.
**Figure 6.** Sucrose gradient fractions and 2D gels of wt and *ffc*. A, Schematic of sucrose gradient fractionation, with molecular weight (MW) cutoff estimations in kDa. F1-monomers and low MW complexes, F2, overlap fraction with Rubisco, F3, Rubisco-containing fraction, F4, high MW fraction. B, 1D SDS-PAGE of each fraction. C, 2D SDS-PAGE of F1 and F2 for both wt and *ffc*.

**Figure 7.** Identification of up-regulated proteins by 2D gel analysis. A, Partial gel image of the corresponding up-regulated spot area for both At1g23740 and At2g28190. Fractions are indicated to the left of the gels. Fractions 1a and 1b correspond to technical replicates of Fraction 1 (F1). Experimental molecular weight (in kDa) is indicated to the right of the gels. B, Bar graph of normalized spot volumes for each protein. Grey bars, wt, white bars, *ffc*. Standard error bars represent a quantification average over 12 gels.

**Figure 8.** Phenotype and mRNA accumulation levels of wt, *clpc1*, *ffc1*, *clpc1 x ffc*. A, 24 day old plants grown on ½ X MS media containing 1% sucrose. wt plants are at stage 1.08, *ffc* and *clpc1* plants are at stage 1.05, *ffc/clpc1* plants are at stage 1.02. The right hand panel shows the seedling lethal phenotype of *clpc1 x ffc* double mutant grown for 30 days on ½x MS media without sucrose. B, RT-PCR of cpSRP54 and CLPC1 for each genotype. *ACTIN* was used as internal control.

**SUPPLEMENTAL INFORMATION**

**Supplemental Table 1.** Identification and annotation of identified proteins in seedlings (A), stroma (B) and thylakoids (C) obtained from chloroplasts isolated from fully developed leaf rosettes.

**Supplemental Table 2.** Quantification data of total leaf seedlings proteomes of *ffc* and wt by iTRAQ.

**Supplemental Table 3.** ICAT based quantification of chloroplast thylakoid proteins from fully developed *ffc* and wt leaf rosettes. (A) All quantified peptides in experiment 1. (B) All quantified peptides in experiment 2. (C) Quantification and integration of data from experiment 1 and 2.
**Supplemental Table 4.** ITRAQ based quantification of chloroplast thylakoid proteins from fully developed *ffc* and wt leaf rosettes.

**Supplemental Figure 1.** Protein to chlorophyll a+b ratios (weight/weight) of cotyledons and leaf pairs 1+2, 3+4, 5+6, 7+8 and 11+12 at developmental stage 1.08 and 1.12 of wt and *ffc*.

**Supplemental Figure 2.** Experimental design of iTRAQ experiments. (A) Scheme of the experiment. The proteins are digested in solution, labeled mixed, fractionated on SCX, followed by identification and quantitation by CapLC-Q-TOF MS. (B) Scheme of the iTRAQ reagent with four different reporter ions. The four different reagents generate an isobaric modification on the peptide in MS. When the peptides are fragmented, reporter ions are generated with m/z 114, 115, 116 and 117. The area of these ions are then compared to determine the relative ratio for the different states. (C) Labeling scheme. To control for biological and experimental variation, two biological replicates each with two labeling replicates were used.

**Supplemental Figure 3.** CN-PAGE of chloroplast stroma from fully developed leaf rosettes of wt and *ffc* plants.

**Supplemental program** for correction of iTRAQ purity.
Fig. 1A,B
(in color)
Fig. 2A,B,C,D
A

|                | wt 1.07 | wt 1.11 | wt 3.90 |
|----------------|---------|---------|---------|
| (development)  | (17d)   | (21d)   | (40d)   |

**Total leaf proteome**

iTRAQ analysis

**Chloroplast proteome**

Stroma ICAT analysis
2DE gel analysis
Thylakoids iTRAQ analysis

B

Fig. 3A,B (in color)
Fig. 4
(in color)
Wt stroma

→ Denature & reduce

Label with cICAT light

(Δ 9Da)

→ Label with cICAT heavy

→ Combine samples

1-D SDS PAGE

12 bands, in-gel digestion trypsin

Extract peptides

→ Avidin Affinity purification

Collect eluate, Cleave biotin tag, Desalt

→ Online-CapLC-ESI-MS for quantification

Online-CapLC-ESI-MS/MS for identification
| pI  | 7  | 4  | 7  | 4  |
|-----|----|----|----|----|
| F1  | F2 | F3 | F4 | F1 |
| 1-300 | 300-550 | 550-800 | > 800 |

**Fig. 6**

A. Gradient gel electrophoresis

B. Protein bands

C. Western blot analysis of wild type (wt) and ffc mutants.
**Fig. 7**

A

|          | BioRepl #1 |          | BioRepl #2 |          |
|----------|------------|----------|------------|----------|
| pl       | 6.0        | pl       | 6.0        | pl       |
| pI       | 6.0        | pI       | 5.8        |          |
| kDa      | -45        | kDa      | -35        |          |
| 1a       |            | 1a       |            |          |
| wt       |            | wt       |            |          |
| ffc      |            | ffc      |            |          |
| 1b       |            | 1b       |            |          |
| wt       |            | wt       |            |          |
| ffc      |            | ffc      |            |          |
| 2        |            | 2        |            |          |

B

**At1g23740**
Oxidoreductase-like protein

|          | Norm. Spot Volume |
|----------|-------------------|
| 1a       | 0.8               |
| 1b       | 0.6               |
| 2        | 0.2               |

**At2g28190**
CuZnSOD

|          | Norm. Spot Volume |
|----------|-------------------|
| 1a       | 2                 |
| 1b       | 1.6               |
| 2        | 1.2               |

**Fig. 7**
Fig. 8 (in color)