Modified Clp Protease Complex in the ClpP3 Null Mutant and Consequences for Chloroplast Development and Function in Arabidopsis1,2,3,4

Jitae Kim, Paul Dominic Olinares2, Soo-hyun Oh, Stefania Ghisaura3, Anton Poliakov4, Lalit Ponnala, and Klaas J. van Wijk*

Department of Plant Biology (J.K., P.D.O., S.-h.O., S.G., A.P., K.J.v.W.) and Computational Biology Service Unit (L.P.), Cornell University, Ithaca, New York 14853

The plastid ClpPRT protease consists of two heptameric rings of ClpP1/ClpR1/ClpR2/ClpR3/ClpR4 (the R-ring) and ClpP3/ClpP4/ClpP5/ClpP6 (the P-ring) and peripherally associated ClpT1/ClpT2 subunits. Here, we address the contributions of ClpP3 and ClpP4 to ClpPR core organization and function in Arabidopsis (Arabidopsis thaliana). ClpP4 is strictly required for embryogenesis, similar to ClpP5. In contrast, loss of ClpP3 (clpp3-1) leads to arrest at the hypocotyl stage; this developmental arrest can be removed by supplementation with sucrose or glucose. Heterothropically grown clpp3-1 can be transferred to soil and generate viable seed, which is surprising, since we previously showed that CLPR2 and CLPR4 null alleles are always sterile and die on soil. Based on native gels and mass spectrometry-based quantification, we show that despite the loss of ClpP3, modified ClpPR core(s) could be formed, albeit at strongly reduced levels. A large portion of ClpPR subunits accumulated in heptameric rings, with overaccumulation of ClpP1/ClpP5/ClpP6 and ClpP3. Remarkably, the association of ClpT1 to the modified Clp core was unchanged. Large-scale quantitative proteomics assays of clpp3-1 showed a 50% loss of photosynthetic capacity and the up-regulation of plastoglobules and all chloroplast stromal chaperone systems. Specific chloroplast proteases were significantly up-regulated, whereas the major thylakoid protease (FtsH1/FtsH2/FtsH5/FtsH8) was clearly unchanged, indicating a controlled protease network response. clpp3-1 showed a systematic decrease of chloroplast-encoded proteins that are part of the photosynthetic apparatus but not of chloroplast-encoded proteins with other functions. Candidate substrates and an explanation for the differential phenotypes between the CLPP3, CLPP4, and CLPP5 null mutants are discussed.

Intracellular proteolysis is essential for proteome homeostasis, the regulation of metabolic and signaling pathways, and, ultimately, the maintenance of organellar and cellular viability. Chloroplasts contain multiple soluble and membrane-bound proteases and processing peptidases (Kato and Sakamoto, 2010; Olinares et al., 2011b) that operate in parallel as well as in series, presumably with partially overlapping substrates. The Clp protease system is the most abundant and complex stromal protease family in the plastid. It consists of five Ser-type Clp proteases (P1 and P3–P6) and four non-proteolytic ClpR subunits (R1–R4), which together constitute the tetradecameric and asymmetric approximately 350-kD Clp protease core formed by two heptameric rings. Furthermore, three Clp AAA+ chaperones (C1, C2, and D) similar to the Escherichia coli ClpA and the adaptor ClpS (homologous to the E. coli ClpS) likely serve to deliver protein substrates to the core complex. Attached to the ClpPR core are ClpT1 and ClpT2, which have similarity to the N-terminal domain of the ClpC/ClpD chaperones (Peltier et al., 2004; Olinares et al., 2011a). These ClpT subunits are unique to chloroplasts and have been hypothesized to regulate chaperone binding and/or substrate selection (Peltier et al., 2004; Olinares et al., 2011b) or to aid in the assembly of the ClpPR core complex (Sjögren and Clarke, 2011).

The quantitative subunit compositions for the intact ClpPR core and each heptameric ring were recently determined (Oлинаres et al., 2011a). The chloroplast ClpP/ClpR protease was affinity purified from clpR4 and clpp3 Arabidopsis (Arabidopsis thaliana) null mutants complemented with C-terminal StrepII-tagged versions of CLPR4 and CLPP3, respectively. The subunit stoichiometry was determined by a mass spectrometry
(MS)-based multiplexed absolute quantification strategy using a concatamer of stable isotope-labeled proteotypic peptides generated from a synthetic gene, also known as the QconCAT approach (Beynon et al., 2005; Pratt et al., 2006). This showed that the ClpPR core consisted of one heptameric ring containing ClpP3, ClpP4, ClpP5, and ClpP6 in a 1:2:3:1 ratio (designated the P-ring) and the other ring containing ClpP1 and ClpR1, ClpR2, ClpR3, and ClpR4 in a 3:1:1:1:1 ratio (designated the R-ring). Moreover, based on biochemical and phylogenetic analysis, it was suggested that ClpT1 and ClpT2 bind to the adaxial side of the P-ring (Olnares et al., 2011a; Sjögren and Clarke, 2011). This Clp core complexity is puzzling and is very different from the much simpler Clp composition in photosynthetic and nonphotosynthetic bacteria. The complexity of the plastid Clp core contrasts also to the homomeric ClpP2 core in plant mitochondria (Peltier et al., 2004) and is specific to higher plants and green algae (Derrien et al., 2012). This suggests a specific adaptation of the ClpPRT protease to the chloroplast proteome, warranting an in-depth analysis.

Genetic and phenotypic analyses of various CLPPR mutants in Arabidopsis showed that each of the tested genes affects embryogenesis or seedling development and chloroplast biogenesis. Interestingly, the severity of the phenotypes for the various CLPPR null mutants differs greatly (for review, see Olnares et al., 2011b). Complete loss of CLPP5 gene expression is embry lethal, whereas complete loss of CLPR2 or CLPR4 delayed embryogenesis and resulted in developmental arrest at the cotyledon stage (Kim et al., 2009). This arrest could be broken by growth on Suc, but seedlings remained sterile (Kim et al., 2009; Olnares et al., 2011b). The Arabidopsis clpr1-1 null mutant could be maintained on soil and produced viable seeds, even if they showed a virulent phenotype (Koussevitzky et al., 2007). Overexpression of CLPR3, but not CLPR2 or CLPR4, in clpr1-1 led to full complementation, indicating that ClpR1 is partially redundant to ClpR3 (Kim et al., 2009). In addition, ClpP1, the only plastid-encoded Clp subunit, was shown to be essential for leaf development in tobacco (Nicotiana tabacum; Shikanai et al., 2001; Kuroda and Maliga, 2003). A mutant in CLPR2 with approximately 20% residual CLPR2 mRNA and ClpR2 protein (clpr2-1; Rudella et al., 2006) and antisense lines against CLPP4 and CLPP6 (Sjögren et al., 2006; Zheng et al., 2006) in Arabidopsis exhibited delayed chloroplast and plant development and a virulent or variegated phenotype.

It is not known if CLPP3, CLPP4, or CLPP6 is essential for embryogenesis, similar to CLPP5. One of the objectives of this study was to address the structural and functional contributions of ClpP3 and ClpP4 and, thus, to complete the genetic analysis of the ClpPR core subunits in Arabidopsis; we note that there are no suitable transferred DNA (T-DNA) insertion mutants for CLPR3 or CLPP6. We will show that null mutants for CLPP4 are embryo lethal, similar to CLPP5, but that null mutants for CLPP3 can germinate, develop seedlings under heterotrophic but not autotrophic conditions, and even produce viable seeds. Thus, in contrast to CLPP4 and CLPP5 null mutants, clpp3-1 is the only null mutant in a ClpP protease subunit that can germinate, likely because other ClpPR subunits can partially substitute for ClpP3. Using the QconCAT technique (Olnares et al., 2011a), we also determined the composition of the ClpPR core in clpp3-1 in an effort to understand how the Clp system can function (even if suboptimally) without ClpP3. Furthermore, we quantified ClpT1/ClpT2 in the various Clp assemblies using immunodetection. Previously, we determined the leaf proteome phenotypes of clpr2-1 (Zybaïlov et al., 2009a) and clpr4-1 (Kim et al., 2009) using MS-based spectral counting methodology. These studies identified approximately 2,800 proteins, and quantification revealed that the strongest effects occurred within the chloroplast, which is consistent with their yellow/pale-green phenotype and delayed growth. A strong loss of the photosynthetic machinery was observed in the leaf proteome of clpr4-1, consistent with its severe albino phenotype and its dependence on exogenous sugar (Kim et al., 2009). Here, we further optimized this large-scale proteomics workflow and developed and implemented a better statistical analysis to determine the molecular phenotype of clpp3-1. Comparison of the proteomics phenotype of clpp3-1 with the clpr2-1 and clpr4-1 phenotypes indicated very similar metabolic and protein homeostasis defects for these Clp core mutants.

RESULTS

ClpP4 Is Essential for Embryogenesis

We screened and genotyped various T-DNA insertion collections with the Columbia-0 background for potential null and knockdown mutants in CLPP4 (At5g45390). This identified one null mutant line (SALK_000913), designated clpp4-1 (Fig. 1A). Developing seeds in siliques of heterozygous plants showed a 3:1 green:white ratio and no abortions, whereas mature siliques showed smaller, darker seeds in the same ratio (Fig. 1B). This is consistent with a single genome insertion in a gene important for plastid development and/or function. None of the progeny of such heterozygous clpp4-1 mutants survived as homozygotes, even when grown under heterotrophic conditions and at low light intensities. Microscopy showed a block in embryogenesis at the (pre)globular stage in the white seeds (Fig. 1C). Heterozygous clpp4-1 had a wild-type phenotype, indicative of recessive alleles without a gene dosage effect (data not shown). clpp4-1 null alleles were recovered in lines transformed with CLPP4 complementary DNA (cDNA) driven by a 1 × 35S promoter, and reverse transcription (RT)-PCR confirmed complementation, further showing that loss of CLPP4 is responsible for the embryo-lethal phenotype (Fig. 1D). Thus, ClpP4 is essential for embryogenesis similar to ClpP5 but unlike the other nucleus-encoded chloroplast Clp core proteins for which null mutants have been tested so far (CLPR1, CLPR2, and CLPR4; Kim et al., 2009).
Loss of ClpP3 Results in Delayed Embryo Development and Seedling Lethality But Can Be Rescued by Adding Sugars to the Growth Medium

We also screened T-DNA insertion collections with the Columbia-0 background for potential null and knockdown mutants in CLPP3 (AT1G66670). This identified one null mutant line (SALK_065330), designated clpp3-1 (Fig. 1A). Similar to clpp4-1, the developing seeds in siliques of heterozygous clpp3-1 plants showed a 3:1 green: white ratio and no abortions, again consistent with a single insertion (Fig. 1B). Microscopy showed delayed embryogenesis, mostly reaching the torpedo stage in the white seeds (Fig. 1C). The homozygous clpp3-1 could be fully complemented with genomic CLPP3 (Fig. 1D) and to a lesser extent also with 1×35S-cDNA-CLPP3-StrepII (Olinares et al., 2011a). PCR of genomic DNA (data not shown) and RT-PCR (Fig. 1D) confirmed the complementation.

Under autotrophic conditions on agar plates, homozygous clpp3-1 seedlings were identified, but their development was arrested at the cotyledon stage and seedlings died after several weeks (Fig. 2A), similar to null mutants in CLPR2 and CLPR4 (Kim et al., 2009). We note that the hypocotyls of clpp3-1 seedlings were clearly greener than clpr2-2 and clpr4-1 null alleles (Fig. 2A). Under heterotrophic conditions on agar plates supplemented with 1% to 2% Suc, homozygous clpp3-1 seedlings developed beyond the cotyledon stage, even if growth and development were slow, and with pale-green serrated leaves (Fig. 2B), as observed previously for CLPR2 and CLPR4 null alleles. The clpp3-1 allele accumulated neither CLPP3 mRNA nor ClpP3 protein (Fig. 2C). clpp3-1 plants grown on agar were greener on 2% Suc than on 1% Suc, and the youngest leaves were paler than the older leaves (Fig. 2D). The chlorophyll and carotenoid concentrations in clpp3-1, on a fresh weight basis, of 7-week-old seedlings grown on agar medium supplemented with 2% Suc were reduced by 77% and 65%, respectively, compared with the wild type (Supplemental Table S1). The total chlorophyll:total carotenoid ratio in clpp3-1 was reduced by 33%.

In many plant studies, Glc is considered primarily a signaling molecule, whereas Suc is primarily considered a source of energy (and used for long-distance transport; Hanson and Smeekens, 2009; Eveland and Jackson, 2012). Therefore, we compared the effects of Suc (1%, 3%, and 5%) and Glc (1%, 3%, and 5%) on seedling development in clpp3-1. For comparison, we also included the leaky clpr2-1 allele, which does not show seedling arrest but has strongly delayed development; this is the Clp core mutant with the strongest
phenotype that can still produce seeds (Rudella et al., 2006). Both Suc (1%–5%) and Glc (1%–5%) broke clpp3-1 seedling arrest for up to 80%, with Glc being somewhat more effective (Fig. 2E). Both sugars strongly stimulated leaf development of clpp3-1 seedlings, as measured by the number of leaves, with an optimum concentration of 3% for both sugars, but leaf formation was faster on Suc (Fig. 2F). In the wild type and clpr2-1, both sugars repressed leaf formation at the higher concentrations (3% and 5%). Glc and Suc showed differential effects on leaf expansion of the wild type, clpp3-1, and clpr2-1, as measured by the rosette diameter (Fig. 2G). For all three genotypes, Suc stimulated rosette diameter, with the largest diameter at 1% Suc for the wild type (16 cm) and clpr2-1 (11 cm) but at 3% Suc for clpp3-1 (10 cm). Rosette diameter was repressed at 3% and 5% Suc for the wild type and clpr2-1. In contrast, Glc showed a concentration-dependent repression of rosette diameter for the wild type and clpr2-1. In the case of clpp3-1, 1% Glc facilitated seedling development by breaking developmental arrest, but higher concentrations repressed leaf growth and expansion, similar to the wild type and clpr2. Increased concentrations of Glc, but not Suc, slightly increased chlorophyll and total carotenoid levels on a fresh weight basis in clpp3-1. Increase of either Glc or Suc levels increased pigment levels on a fresh weight basis in the wild type and clpr2-1, presumably by reduced cell expansion and unaffected pigment accumulation (Supplemental Fig. S1).

The clpp3-1 Null Allele Can Flower and Produce Viable Seeds, Unlike the Other Seedling-Lethal ClpPR Core Mutants

After several weeks on Suc, clpp3-1 seedlings started to green and accumulate chlorophyll, and surprisingly, they could be transferred to soil, eventually flowered, and generated viable seeds (Fig. 3), unlike the CLPR2 and CLPR4 null alleles, which died soon after transfer.
to soil (Kim et al., 2009). Chlorophyll and carotenoid levels of these clpp3-1 plants were about 50% lower than in wild-type plants (Supplemental Table S1). Thus, CLPP3 is different from CLPR2, CLPP4, CLPP5, and CLPR5, indicating that CLPP3 is not strictly essential once chloroplasts and leaves have reached the mature stage. This suggested that other CLP-P/CLP-R subunits partially compensate for the loss of ClpP3 (even if very poorly and only late in development). Because in wild-type Columbia-0 plants, ClpP3 is only found in the P-ring with other nucleus-encoded ClpP subunits (ClpP4-ClpP6), it seemed most logical that these nucleus-encoded ClpP subunits substitute for ClpP3. This would be similar to the replacement of ClpR1 by ClpR3 in the R-ring, as we showed previously through overexpression of CLP3 cDNA in the clpr1-1 background (Kim et al., 2009). Therefore, we tested if overexpression of CLPP4, CLPP5, or CLPP6 could complement the clpp3-1 mutants. clpp3-1 heterozygous plants were transformed with CLPP4, CLPP5, or CLPP6 cDNAs using both 2×35S promoters (pMD32 binary vector) and 1×35S promoters (pEARLYGATE100) and the cDNA for CLP3 as a positive control. Numerous complemented clpp3-1 lines with CLPP3 StrepII-tag cDNA were recovered, in addition to complemented lines with genomic DNA (Fig. 1D). Despite these extensive efforts, no complementation was observed for overexpression with CLPP4, CLPP5, or CLPP6. This suggests that these nucleus-encoded ClpP proteins by themselves cannot functionally substitute for ClpP3, but overexpression of multiple CLP-Ps together might be able to complement clpp3-1, as our biochemical analysis of Clp complexes in clpp3-1 seems to suggest (see below).

Synergistic Genetic Interactions between CLPP3 and CLPR2

To explore the relationship between the P- and R-rings of the ClpPR core complex, we investigated the genetic interaction between clpp3-1 and clpr2-1. The double homozygous clpp3-1 × clpr2-1 mutant was recovered from plants grown under heterotrophic conditions at low light (Fig. 4A) but was not viable when transferred to soil. This double mutant was extremely reduced in growth and development and remained yellow to very pale green and never produced flowers, even under the most favorable conditions (i.e. low light and 1%–2% Suc). Thus, clpp3-1 and clpr2-1 have a synergistic effect.

Interestingly, we observed a clear gene dosage effect of CLP3 (heterozygous clpr2-1) on the homozygous clpr2-1 mutant. The double mutant homozygous for the clpr2-1 allele but heterozygous for the clpp3-1 allele (Aabb) is shown in Figure 4B. This double mutant has a stronger phenotype than clpr2-1 but can be still grown on soil and produces viable seeds. The double mutant homozygous for the clpp3-1 allele but heterozygous for the clpr2-1 allele (aaBb) has the same phenotype as the clpp3-1 single mutant (data not shown). RT-PCR confirmed the reduced mRNA for CLPR2 in the clpr2-1 background and complete loss for CLP3 in the double homozygous mutant. A partial reduction for CLP3 was observed in heterozygous clpp3-1 in the homozygous clpr2-1 background (Fig. 4C). Furthermore, CLPR2 mRNA levels were influenced by the levels of CLP3 mRNA. It can thus be concluded that there is an interaction effect between the expression/accumulation of subunits of the P- and R-rings, similar to subunits within the R-ring (when crossing ClpR1 and ClpR2 alleles; Kim et al., 2009).

Accumulation and Assembly State of ClpPRT Subunits in clpp3-1

To determine if other Clp subunits are up-regulated to compensate for the loss of ClpP3, or otherwise affected, we determined accumulation levels for the ClpPR core proteins ClpR2, ClpP3, ClpP4, and ClpP6, peripheral subunits ClpT1 and ClpT2, the substrate regulator ClpS, and the Clp chaperones ClpC1 and ClpC2 using immunoblotting. Total leaf proteins were extracted from clpp3-1 plants transferred to soil as well as wild-type plants at a comparable developmental stage. SDS-PAGE followed by immunoblotting showed that the accumulation level of ClpR2 was unchanged in the clpp3-1 mutant, whereas accumulation levels of ClpP4, ClpP6, ClpT1, and ClpT2 were 2- to 3-fold up-regulated in clpp3-1 (Fig. 5). The ClpS level increased 4-fold in clpp3-1. Moreover, ClpC1 and ClpC2 were both down-regulated by 30%. Interestingly, in clpp3-1, most of ClpT1, but not ClpT2, showed an approximately 1-kD upward mass.
shift due to an unknown posttranslational modification or modified processing (Fig. 5).

To determine the consequences of the loss of ClpP3 on the assembly state of the ClpPR complex and the association of ClpT, chloroplast soluble proteomes (stroma) of the wild type and clpp3-1 were extracted under nonnondenaturing conditions, and proteins were separated by one-dimensional native gel electrophoresis. For comparison, we also included the clpr2-1 mutant in the analysis. In the wild type, ClpR2, ClpP4, and ClpP6 were mostly observed in the expected 350-kD tetradecameric ClpPRT complex (Fig. 6). However, some signal (in particular for ClpR2) was also detected in a band around 200 kD, corresponding with single heptameric rings; this reflects destabilization of the tetradecameric at the heptamer interface, similar to our previous observations (Olinares et al., 2011a; Fig. 6). In the case of clpr2-1, the total signal for ClpR2 was reduced, which is consistent with the 5-fold reduced expression in clpr2-1 reported previously (Rudella et al., 2006). Similar to the wild type, ClpR2 in clpr2-1 was mostly found in the 350-kD complex, with lower amounts accumulating in the 200-kD heptamers. In contrast, in clpp3-1, nearly all of ClpR2 and ClpP4 were found around 200 kD, with only small amounts accumulating between 350 and 400 kD. It also appears that this complex was found at 50 kD lower than the 350-kD band observed in the wild type and clpr2-1. ClpP4, but not ClpR2, was found in two bands in the 200-kD region. For both clpr2-1 and clpp3-1, about 50% of ClpP6 accumulated in 350-kD complexes, with the remainder of ClpP6 found in two separate bands in the 200-kD region. In the case of ClpT1, essentially all protein was found in the 350-kD complex for the wild type, clpr2-1, and clpp3-1. A very small amount (less than 5%) of ClpT1 was also found in a single band around 200 kD in the case of clpr2-1 and clpp3-1. The signals for ClpT2 in the 200- to 400-kD region were very weak and therefore inconclusive, perhaps because the vast majority of ClpT2 accumulated as free monomers or dimers (data not shown).

Together, these results show that in the wild type, a single ClpPRT complex accumulates as expected, with some destabilization occurring, resulting in an accumulation of approximately 200-kD heptameric rings. Previously, we showed by affinity tagging and MS analysis that these heptameric rings contained either ClpP1, ClpR1, ClpR2, ClpR3, and ClpR4 or ClpP3, ClpP4, ClpP5, and ClpP6 (Olinares et al., 2011a). In the case of clpp3-1, only ClpP6 and ClpT1, but not ClpP4 and ClpR2, accumulated at high levels in 350- to 400-kD Clp core complexes. In clpp3-1 and clpr2-1, ClpP4 and ClpP6 were found in two complexes in the 200-kD region. The nature of this heterogeneity in the 200-kD region is not clear.

Stoichiometry Determination of Clp Assemblies in clpp3-1

To further determine the composition and assembly state of the ClpPR complex in clpp3-1, and possibly to
determine if one or more Clp subunits compensate for ClpP3 in the assembled core, we implemented the QconCAT approach on chloroplast stroma, but now without StrepII-affinity purification of the Clp assemblies (Fig. 7). This is more challenging because of the higher complexity of the samples, but it was not practically feasible to create such StrepII-tagged Clp lines in the clpp3-1 background. Chloroplast stromal samples from the wild type and clpp3-1 were separated by native gel electrophoresis, and the region between 150 and 400 kD was cut into four gel slices, such that it would capture the intact ClpPRT cores (250- to 400-kD range) and the individual heptameric rings (150- to 250-kD range; Fig. 7). Proteins in each gel slice were digested with trypsin, and the resulting peptide mixtures were spiked with the in-gel-digested stable isotope-labeled Clp-QconCAT standard followed by liquid chromatography (LC)-MS analysis using an LTQ-Orbitrap. The absolute amount of each Clp subunit was determined from the measured sample-to-standard peak area ratios of the representative peptides. The MS analysis was carried out in triplicate. This work follows is summarized in Figure 7.

ClpP5, ClpP6, ClpR2, and ClpR4 could be quantified by two distinct peptides each, whereas the amounts of
ClpP1, ClpR1, and ClpR3 could be measured by one peptide each (Supplemental Table S2). ClpP4 could not be quantified, since the standard peptides could not be detected due to the relatively high sample complexity. However, we detected ClpP4 from large-scale proteomic analyses of total leaf extracts from *clpp3-1* (see below), and we determined accumulation levels and assembly state from immunoblotting (Figs. 5 and 6); this allowed us to consider ClpP4 as a candidate for compensation of the loss of ClpP3 (see “Discussion”). From the molar amount of each Clp subunit per gel band, the relative distribution of the Clp subunits across various assembly states could be determined (Fig. 8). To simplify the calculations and outcomes, and because the stoichiometries between the Clp subunits were similar between the two gel slices within the 250- to 400-kD range (bands 1 and 2; the Clp core complexes) and also within the 150- to 250-kD range (bands 3 and 4; the heptameric rings), we pooled the data within each of these mass ranges (Supplemental Table S2). Figure 8, A and B, shows the molar distribution of the Clp subunits across the Clp cores and P- and R-rings, whereas Figure 8C shows the overall distribution.

In the wild type, 54% of the Clp subunits were detected in the Clp core complex, with the remaining 46% accumulating in heptamers. In contrast, in *clpp3-1*, only 12% of ClpPR subunits assembled in the Clp cores, with the remaining 88% in Clp heptameric rings (150–250 kD). This indicates that Clp subunits in *clpp3-1* could still associate into Clp rings, but Clp core assembly formation was inefficient, in agreement with the immunoblotting results (Fig. 6).

The asymmetric Clp core complex is composed of the P-ring and the R-ring in a 1:1 stoichiometry and an overall ClpPR stoichiometry for [P3:P4:P5:P6];[P1:R1:R2:R3:R4] of [1:2:3:1]:[3:1:1:1:1] (Oliñares et al., 2011a). To evaluate the stoichiometry of ClpPR proteins in the Clp core complex, we chose ClpR4 as a normalizer, because it could be reliably quantified with two peptides (for details and *sd* values, see Supplemental Table S2). For the Clp core complex in wild-type stroma (250–450 kD), the molar ratio for [P3:P5:P6]:[P1:R1:R2:R3:R4] was [1:3:1]:[3:1:1:1:1] (Table I), which was identical to what we observed with the affinity-purified Clp core (Oliñares et al., 2011a), except that we could not quantify ClpP4. Moreover, the ClpPR stoichiometries in the mixture of individual heptameric rings (between 150 and 250 kD) in the wild type were similar in the core (Table I). In contrast, the ClpPR stoichiometries in the Clp core of *clpp3-1* were altered with increased numbers of ClpP5, ClpP1, and ClpR3 (and complete loss of ClpP3), resulting in an approximate ratio of [0:4:1]:[5:1:1:2:1] for [P3:P5:P6];[P1:R1:R2:R3:R4], which suggest mixing of the subunits of the R- and P-rings. Similarly, also in the mixture of individual heptameric rings (between 150 and 250 kD), the ratio was changed, now with even stronger overrepresentation of ClpP5 (Table I). Summation of the data for the core and the rings resulted in the expected stoichiometry for the wild type but increased copy numbers for ClpP5, ClpP1, ClpR3, and to a lesser degree ClpP6 for *clpp3-1* (Fig. 8C; Table I). Absolute amounts of ClpR1, ClpR2, and ClpR4 (normalized to total stromal proteins) were unchanged in *clpp3-1* as compared with the wild type, whereas there was 2–2.5-fold more ClpP1, ClpP5, ClpP6, and ClpR3 (Fig. 8C).

**Figure 8.** Distribution of Clp subunits among the high-molecular-mass Clp assemblies for the wild type (wt) and *clpp3-1*. The molar amounts of each Clp subunit across mass ranges 250 to 450 kD and 150 to 250 kD were determined using the QconCAT approach as outlined in Figure 7. Subunits were grouped as components of the P-ring (ClpP3, ClpP4, ClpP5, and ClpP6) and the R-ring (ClpP1, ClpR2, ClpR3, and ClpR4). ClpP4 could not be quantified. Absolute amounts of ClpPR subunits (in fmol) in the mass range 250 to 450 kD corresponding to ClpPR core complexes (A), in the mass range 150 to 250 kD corresponding to heptameric rings (B), in the combined samples (across 150 to 450 kD; C) are shown. Values above the bars indicate the *clpp3-1*: wild-type ratio for each protein. *sd* values are indicated.

**Phenotypic Analysis of clpp3-1 by Comparative Quantitative Proteomics**

To gain insight into the consequences of the loss of CLPP3, we compared the total denatured leaf proteomes of *clpp3-1* and wild-type rosettes (Fig. 9). Leaf rosettes were obtained from pale-green *clpp3-1* plants (initially grown in heterotrophic conditions and then transferred to soil) and soil-grown wild-type plants.
Supplemental Table S3). This resulted in the identification of 2,313 proteins, quantified in 1,993 individual MS/MS spectra; proteins were identified, and quantified MS data were searched and filtered (Supplemental Fig. S2; Table I). Each gel lane was excised in 20 slices, followed by in-gel trypsin digestion and protein identification by nano-LC-tandem mass spectrometry (MS/MS). Three closely related proteins that were identified with pep-ClpPR complexes using StrepII-ClpR4- and StrepII-P3-complemented Arabidopsis null mutant lines. Table I. Stoichiometry of Clp subunits determined by MS analysis and the QconCAT technique

| Subunit | Core<sup>4</sup> | Ring<sup>3</sup> | Core + Rings | Core<sup>2</sup>
|---------|-----------------|----------------|--------------|-------------
| ClpP3   | 1.0             | 0.6            | 0.8          | 1.0         |
| ClpP4   | nd              | nd             | nd           | 2.4         |
| ClpP5   | 3.4             | 3.4            | 3.4          | 7.3         |
| ClpP6   | 0.9             | 1.4            | 1.5          | 11.1        |
| Sum for P-ring | 5.3 | 5.3 | 9.5 | 8.8 |
| ClpP1   | 2.9             | 4.9            | 5.5          | 3.1         |
| ClpR1   | 1.0             | 0.6            | 1.0          | 1.2         |
| ClpR2   | 0.8             | 0.4            | 0.9          | 1.1         |
| ClpR3   | 0.8             | 1.2            | 1.9          | 1.0         |
| ClpR4   | 1.0             | 1.0            | 1.0          | 1.0         |
| Sum for R-ring | 6.5 | 8.8 | 7.5 | 10.2 |

<sup>4</sup>From the 450- to 250-kD mass range.  <sup>3</sup>From the 250- to 150-kD mass range.  <sup>2</sup>Affinity-purified ClpPR complexes using StrepII-ClpR4- and StrepII-P3-complemented Arabidopsis null mutant lines.

To determine which proteins/protein groups were differentially expressed in *clpp3-1* relative to the wild type, two statistical tools, QSpec (Choi et al., 2008) and GLEE (A. Poliakov, L. Ponnala, P.D. Olinares, and K.J. van Vijk, unpublished data), were employed. These tools were specifically developed for significance analysis of large-scale spectral counting experiments, and both have their merits (for discussion, see Supplemental Text S1).

Expression and MS/MS analyses of the total leaf data sets using QSpec (Bayes factor > 10) and GLEE (P < 0.01). A total of 148 of the plastid-localized proteins and 64 extraplastidic proteins passed both statistical tests (Supplemental Table S3). These extraplastidic proteins were located in diverse subcellular compartments and did not show any functional trends; this indicates the lack of a specific extraplastidic phenotype in *clpp3-1*. In the remainder of this paper, therefore, we will focus on the effects on the plastid proteome and the differentially accumulating plastid proteins (Table II). Immunoblot analysis for a number of thylakoid and stromal proteins was consistent with the results of the MS-based quantification, providing further support for the reliability of MS-based quantification and statistics (Fig. 10). To evaluate general effects of the loss of ClpP3 on the plastid, we compared the chloroplast protein mass investments (based on the normalized adjusted spectral counts [NadjSPC] of designated chloroplast proteins) across 35 functions between *clpp3-1* and the wild type (Fig. 11).

In the next sections, we discuss the results of the proteome analysis in an effort to (1) determine the molecular chloroplast phenotype of *clpp3-1*, (2) identify potential substrates among the up-regulated proteins, and (3) compare the results with previous quantitative proteome analyses *clpr2-1* (Zybailov et al., 2009a) and *clpr4-1* (Kim et al., 2009) to look for consistency and differences between ClpPR core mutants. We note that, due to the improved experimental workflow and MS acquisition settings, we quantified substantially more proteins in *clpp3-1* than in *clpr2-1* and *clpr4-1* (for more information
on how this improvement was accomplished, see Supplemental Text S1; Supplemental Fig. S3; Supplemental Table S5).

Reduced Photosynthetic Capacity in clpp3-1

About 100 proteins involved in the dark and light reactions of photosynthesis were quantified. The overall protein mass of the thylakoid-bound photosynthetic apparatus was decreased by 30% (Fig. 11A). Nearly 40 proteins in the thylakoid photosynthetic apparatus were significantly down-regulated (Table II). These included proteins of each of the five thylakoid complexes (PSI and PSII, cytochrome b6f, ATP synthase, and NADH dehydrogenase [NDH]). An interesting exception was LHCI-5, which was nearly 5-fold up-regulated. LHCI-5 is a low-abundance protein shown to loosely associate with PSI (Ganeteg et al., 2004) and interacts with the light-harvesting complex I at the Lhca2/Lhca3 site (Lucinski et al., 2006). The NDH-PSI supercomplex was absent in knockout lines of LHCI-5, indicating a potential role for LHCI-5 in mediating the NDH and PSI association (Peng et al., 2010). Related, stromal CRR6 (for chlororespiratory reduction6), involved in assembly of the NDH complex (Munshi et al., 2006), was approximately 4-fold up-regulated (Table II). The overall mass of the Rubisco complex was decreased by 40% (Fig. 11A), consistent with the visible loss of Rubisco subunits on the Coomassie-stained gel (Fig. 9B). Indeed, down-regulation of the small and large subunits of Rubisco was statistically significant (Table II). Surprisingly, the mass of the rest of the Calvin cycle enzymes was not affected in clpp3-1 (Fig. 11A), and none of the individual proteins was significantly affected, with the exception for one of the isoforms of fructose bisphosphate aldolase (SFBA1; At2g21330), which was 50% up-regulated. SFBA1 was found associated in significant amounts with plastoglobules (PGs; Kessler and Schnell, 2006; Ytterberg et al., 2006), which may explain its up-regulation.

Up-Regulation of the PG Proteome Indicates a Stressed Chloroplast in clpp3-1

PGs are thylakoid-associated lipoprotein particles that serve as compartments for the synthesis, storage, and degradation of prenyl lipids and thylakoid membrane remodeling (Bréhélin et al., 2007; Lundquist et al., 2012). The total mass of PG proteins increased 2.6-fold (Supplemental Table S4). As structural proteins, fibrillins maintain the PG coat and likely control PG size, but they may have metabolic functions (Singh and McNellis, 2011). Out of the seven PG-localized fibrillins, three (FBN1a, FBN1b, and FBN8) were significantly 3- to 15-fold up-regulated in clpp3-1 (Fig. 11B). PG-localized metabolic enzymes NADH dehydrogenase, carotenoid dioxygenase, and ABC1 kinase3 were also strongly up-regulated in clpp3-1 (Fig. 11B). PG-localized metabolic enzymes NADH dehydrogenase, carotenoid dioxygenase, and ABC1 kinase3 were also strongly up-regulated in clpp3-1 (Fig. 11B). This is in striking agreement with coexpression analysis based on mRNA abundance, which showed that these three PG enzymes were part of the same coexpression module as the ClpPR core proteins (Lundquist et al., 2012).

Effects on Plastid Gene Expression and Protein Homeostasis

Figure 11B summarizes the effects on plastid gene expression and protein homeostasis. Proteins (in total,
Table II. Chloroplast-localized proteins that are significantly up-regulated or down-regulated in clpp3-1 plants relative to the wild type

Chloroplast-localized proteins that passed the significance analyses for both QSpec (Bayes factor $>10$) and GLEE ($P < 0.01$) at the 5% false discovery rate threshold are shown. Subplastidial location: E, envelope; IE, inner envelope membrane; IES, inner envelope membrane associated, stroma side; L, lumen; S, stroma; TI, thylakoid membrane, integral bound; TL, thylakoid membrane associated, lumenal side; TS, thylakoid membrane associated, stroma side.

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(Table continues on following page.)
268) involved in these processes were grouped in 11 functional classes. This showed that overall protein mass investments in plastid ribosomes, the Clp system, protein assembly, and DNA-related functions were unchanged in clpp3-1. In contrast, overall protein mass investments in protein (un)folding, tRNA synthases, protein synthesis, and other proteolytic systems were 2-fold increased. Furthermore, protein-targeting machinery and proteins involved in RNA metabolism also overaccumulated in clpp3-1 (Fig. 11B). This general pattern was also reflected in the significantly affected proteins across these functions (Table II). More than 40 proteins were significantly affected, all of which were up-regulated, except for a lumenal isomerase (consistent with the reduction of the lumenal proteome) and three 50S ribosomal proteins, which were down-regulated. The up-regulated proteins include the chloroplast chaperones CPN60a,b (Fig. 10), CPN10,20, cpHSP70-1,2, and cpHSP90, which play crucial roles in protein folding and maturation (Boston et al., 1996; Wandinger et al., 2008). ClpB3, the chloroplast homolog of the bacterial ClpB protein (Myouga et al., 2006), was 5-fold up-regulated. ClpB3 is involved in unfolding aggregated proteins together in the cpHSP70 system (Goloubinoff et al., 1999; Haslberger et al., 2008). The up-regulation of these chaperone systems was also observed in other Clp mutants (Rudella et al., 2006; Kim et al., 2009; Zybailov et al., 2009a). ROC4, an abundant stromal peptidylprolyl isomerase (Peltier et al., 2006; Zybailov et al., 2008) with in vitro rotamase activity (Lippuner et al., 1994), also overaccumulated. In summary, loss of ClpP3 accumulation clearly resulted in major protein-folding stress, perhaps due to the accumulation of unwanted proteins.

Out of the 25 other detected proteases, just three stromal proteases/peptidases and two thylakoid proteases were significantly affected in clpp3-1. Stromal Zn2+ -protease PreP1, stromal processing peptidase (SPP), and leucyl aminopeptidase2 (LAP2) were 2.4- to 5.5-fold up-regulated (Table II). PreP1 was suggested to be involved in the degradation of cleaved chloroplast transit peptides (Bhushan et al., 2005; Stähl et al., 2005; Glaser et al., 2006), whereas stromal SPP is involved in the transit peptide removal of most nucleus-encoded, chloroplast-targeted proteins (Richter and Lamppa, 1998, 1999). LAP2 belongs to the family of soluble aminopeptidases (Walling, 2006), and it was recently suggested that LAP2 also moonlights as a chaperone in the chloroplast (Scranton et al., 2012). The two affected thylakoid proteases are EGY2 and SPPA, which are 3- and 6-fold increased, respectively, in clpp3-1. EGY2 is an ATP-independent intramembrane protease in the Site-2 protease family (Chen et al., 2012) and is a paralog of the...
better-characterized EGY1 thylakoid-bound metalloprotease that is crucial for thylakoid development and the accumulation of chlorophyll-containing proteins (Chen et al., 2005). SppA is a light stress-induced, thylakoid-bound, ATP-independent Ser-type protease with unknown substrates (Lensch et al., 2001). Interestingly, the very abundant thylakoid FtsH protease complex (FtsH1, FtsH2, FtsH5, and FtsH8; Liue et al., 2010b; Kato et al., 2012), robustly quantified with many MS/MS spectra (114, 499, 528, and 146 for FtsH1, FtsH2, FtsH5, and FtsH8, respectively; Supplemental Table S3), was not significantly affected (between 0.8- and 1.2-fold). Immunoblot analyses confirmed the fold changes of SppA, whereas the abundance levels of ClpR2 and the thylakoid protease FtsH2 were unchanged, consistent with the MS-based quantifications (Fig. 10). Thus, the loss of Clp protease capacity specifically affects a subset of chloroplast proteases, indicative of a controlled protease network.

pTAC11 (WHY3) and pTAC17, initially identified in transcriptionally active chromosome (TAC) fractions (Pfalz et al., 2006), were 7- and 3-fold up-regulated in clpp3-1, respectively. In particular, pTAC11, but not pTAC17, is strongly enriched in nucleoids (Majeran et al., 2012; Huang et al., 2013). The function of pTAC17 is unknown, but sequence analysis suggests that it may be involved in DNA repair; importantly, we recently identified it as a candidate substrate for the substate selector ClpS (K. Nishimura, Y. Asakura, G. Friso, J. Kim, S.H. Oh, H. Rutschow, L. Ponnala, and K.J. van Wijk, unpublished data). Plastid pTAC11 is a member of the Whirly family of multifunctional RNA- and DNA-binding proteins located in mitochondria and plastids (Krause et al., 2005). Members of the Whirly family are involved in organelle genome stability and quality control and, perhaps, RNA metabolism (Prikryl et al., 2008; Maréchal et al., 2009; Cappadocia et al., 2012). A SET domain-containing protein (At5g14260) was also significantly overaccumulating in clpp3-1; the SET domain is frequently found in DNA-interacting proteins. Finally, a DEAD box DNA helicase likely involved in DNA folding and YMLG1 were up-regulated. YMLG1 (Kabeya et al., 2010) is involved in the distribution of nucleoids in chloroplast. Together, these results suggest that the loss of Clp protease function has an impact on the plastid chromosome.
These include PPR and RRM proteins, RNase RNase J and an uncharacterized RNase, as well as three DEAD/DEAH RNA helicases, including RH22, RH3, and uridylyl kinase (DPT1). Bacterial RNase J has been implicated in 16S ribosomal RNA maturation and ribosome assembly (Britton et al., 2007), and the Arabidopsis RNase J is essential for embryogenesis (Meinke et al., 2009). Recently, we characterized RNA helicase3 (RH3) as a plastid RNA splice factor, also affecting ribosome biogenesis (Asakura et al., 2012). PPR protein SVR7 was identified as a suppressor of the FTSH2 protease VAR2 and has a chloroplast ribosomal RNA phenotype (Liu et al., 2011a). Uridylate kinase is involved in post-transcriptional steps of psaA/psaB transcript accumulation (Hein et al., 2009). Thus, loss of Clp protease function also impacts RNA metabolism.

Protein elongation factors EF-Tu, EF-G, TypA/bipA (SVR3), three tRNA synthetases, and the Arabidopsis ortholog of bacterial trigger factor were several fold up-regulated (Table II). E. coli trigger factor binds to the 70S exit tunnel and prevents misfolding and aggregation of emerging nascent proteins (Ferbitz et al., 2004). This indicates that loss of Clp protease function leads to a bottleneck in plastid translation.

No Systematic Defect in the Accumulation of Plastid-Encoded Proteins

To determine if clpp3-1 suffered from a systematic problem in the accumulation of chloroplast-encoded proteins, we evaluated the accumulation of chloroplast-encoded proteins. In total, 53 chloroplast-encoded proteins were identified and constituted 34% of the total chloroplast proteome mass in the wild type but only 23% in clpp3-1. Only chloroplast-encoded proteins that are part of the photosynthetic apparatus (25 proteins) were down-regulated, likely reflecting a systematic down-regulation of the photosynthetic apparatus, including nucleus-encoded proteins, rather than a general defect in plastid gene expression. Indeed, accumulation levels of chloroplast-encoded proteins that are not involved in photosynthesis (in total, 28 proteins), including ribosomal proteins, the PEP complex, YCF1 and YCF4, ClpP1, and carboxyltransferase β (part of ACCase), were unchanged (or even up-regulated) in clpp3-1. This is an important conceptual result.

Carbon Metabolism

Figure 11C shows the mass investments in primary carbon metabolism, including the metabolism of starch and minor carbohydrates, photorespiration, glycolysis, the oxidative pentose phosphate (OPP) pathway, and organic transformation (dominated by the abundant carbonic anhydrases and malate dehydrogenase). Likely in response to the loss of photosynthetic capacity, the OPP pathway was 2.6-fold up-regulated in clpp3-1; indeed, two OPP enzymes significantly increased in clpp3-1 (Table II). Investment in the metabolism of various minor carbohydrates and well as starch metabolism were also up-regulated (Fig. 11C). Consistently, six enzymes in starch synthesis and degradation were significantly up-regulated, in agreement with observations for the other Clp core mutants, clpr2-1 and clpr4-1 (Table II). It is not clear why starch metabolism is up-regulated, but it is perhaps in response to the shortage of reduced carbohydrates produced by the chloroplast.

Strong Up-Regulation of ATP Transporters Suggests That the Chloroplast Is Starved for ATP

Four envelope metabolite transporters were up-regulated in clpp3-1 (Table II), of which the two nucleoside triphosphate transporters (NTT1 and NTT2) are involved in importing ATP (Reinhold et al., 2007). These NTT isoforms were 6- to 10-fold up-regulated in clpp3-1, which is consistent with the strong loss of photosynthetic capacity.

Responses of Other Chloroplast Metabolic Pathways to the Loss of ClpP3

Figure 11D summarizes the investment of other functions in the chloroplast. Investments in metal homeostasis and the function plastid division and signaling were doubled in clpp3-1. Specifically, metal homeostasis included three ferritins involved in the storage of iron and two copper chaperones (CCS and CUTA). Up-regulation (4-fold) of ferritin1 was significant (Table II). The increased abundance in plastid division was in particular due to increased levels of the FtsZ family. Investments in several other metabolic pathways (metabolism of fatty acids, isoprenoids, nucleotides, nitrogen, sulfur, and amino acids) were increased by 50%. Consistently, several dozen proteins in these functions were significantly up-regulated, in particular, proteins involved in fatty acid and amino acid metabolism (Table II). In contrast, investment in tetrapyrrole metabolism (chlorophyll, heme, and siroheme) was unchanged in clpp3-1 (Fig. 11D); indeed, only one protein in tetrapyrrole metabolism was affected in clpp3-1 (Table II). NADH-GOGAT/GLT1, mostly involved in the assimilation of nitrogen, stands out for its very strong (22-fold) up-regulation. Ferredoxin-GOGAT, primarily involved in the generation of Glu to support the assimilation of photorespiratory ammonium, was also significantly up-regulated in clpp3-1 (Table II). 4-Hydroxy-3-methylbutyl diphosphate synthase (HDS) controls the methylenylthriol phosphate pathway that generates precursors for plastid isoprenoids. HDS was up-regulated in clpp3-1, in agreement with the other Clp core mutants, clpr2-1 (Rudella et al., 2006) and clpr4-1 (Kim et al., 2009). Interestingly, methylenylthriol cyclodiphosphate, the substrate of HDS, elicits the expression of selected stress-responsive nucleus-encoded plastid proteins (Xiao et al., 2012).
Comparison of clpp3-1 with clpr2-1 and clpr4-1 Showed Exceptional Consistency in Proteome Phenotypes

Previously, we carried out comparative proteome analysis of leaf extracts of the clpr2-1 and clpr4-1 core mutants. Because the ClpPR subunits assemble in a single functional complex, we postulated that it is likely that the molecular phenotypes should be very similar. Therefore, we determined if the significantly altered proteins in clpp3-1 were also affected in these core mutants. Indeed, respectively, 36 and 40 proteins were also significantly affected in these mutants, and importantly, for all but one (AOS), these proteins showed similar direction of response (up or down) to clpp3-1. This shows that the molecular plastid phenotypes of the three ClpPR core mutants are very similar and also underscores that our workflow was very robust and that our significance analysis did not produce many false positives.

DISCUSSION

Functional and Structural Contributions of ClpPR Subunits to the Chloroplast ClpPR Core Complex

The presence of an extended family of four non-catalytic ClpR proteins and five catalytic ClpP proteins in higher plant chloroplasts suggests specific evolutionary adaptation of the Clp protease system to higher plant plastid/chloroplast metabolism and protein homeostasis. Therefore, in an effort to unravel this adaptation, we and others have aimed to establish the functional and structural contributions of each of the nine different ClpPR subunits to the plastid Clp protease in Arabidopsis (for review, see Olinares et al., 2011b). The tetradecameric Clp protease core consists of the heptameric R-ring with ClpP1, ClpR1, ClpR2, ClpR3, and ClpR4 in a 3:1:1:1:1 ratio and one heptameric P-ring with ClpP3, ClpP4, ClpP5, and ClpP6 in a 1:2:3:1 ratio (Olinares et al., 2011a). The stoichiometry and distribution of the ClpPR subunits across and within these two heptameric rings provide an excellent basis for understanding the contribution of each subunit.

Including this study, null mutants for six of the eight nucleus-encoded ClpPR subunits (R1, R2, R4, P3, P4, and P5) have now been obtained and characterized (Koussevitzky et al., 2007; Kim et al., 2009). Moreover, chloroplast-encoded ClpP1 was shown to be essential for shoot development in tobacco (Shikanai et al., 2001; Kuroda and Maliga, 2003). In addition, antisense lines for ClpP4 (Zheng et al., 2006) and for ClpP6 (Sjögren et al., 2006) were investigated that could germinate, develop on soil, and produce seeds; these plants still expressed ClpP4 or ClpP6, albeit at low levels. Down-regulation of ClpP4 or ClpP6 resulted in reduced greening, reduced photosynthesis, and delayed development, as expected. In the case of antisense ClpP6, using SDS-PAGE gels and immunoblotting did not show consistent changes in ClpPR accumulation levels or in systematic differences between the subunits of the R-ring and the P-ring (except of course for reduced ClpP6; Sjögren et al., 2006). Currently, ClpR3 is the only ClpPR subunit for which no mutant has been described. This subunit is interesting, as it can fully complement the ClpR1 null mutant when ClpR3 is overexpressed (Kim et al., 2009), and ClpR3 protein levels increased in the clpr1-1 mutant background (Stanne et al., 2009).

Taking this organization and all other experimental data, as well as the primary sequences for the ClpPR proteins, into account, we can describe the following emerging view of the Clp protease core. Both the non-catalytic ClpR and catalytic ClpP subunits make important contributions to Clp core functions with very little structural or functional redundancy. Within the R-ring, ClpR3, but not the other ClpR proteins, can partially substitute for ClpR1. Loss of ClpP1, ClpR2, or ClpR4 results in dramatic chloroplast and leaf developmental phenotypes and is strictly required for flowering, whereas also embryo plastid development is negatively impacted. Within the P-ring, the subunits present in more than one copy (ClpP4 and ClpP5) are absolutely required for embryogenesis, whereas the function of ClpP3 present in one copy is not strictly required for embryogenesis but is strictly required for leaf development and flowering. However, supplementation with sugars (Glc or Suc) can suppress part of the developmental phenotype, unlike in mutants for members of the R-ring (ClpR2 and ClpR4). Whereas antisense lines of ClpP6 show that this subunit is important for chloroplast biogenesis and leaf development, it is not known if null alleles in ClpP6 phenocopy the ClpP3 null allele or the ClpP4 and ClpP5 null alleles. However, given that ClpP6 is present in only one copy, we speculate that a null allele would resemble the ClpP3, ClpR2, or ClpR4 allele.

The Assembly State of the Clp Core without ClpP3

Our results indicate that in clpp3-1, a much smaller percentage of ClpPR proteins can effectively assemble into 350- to 400-kD core complexes. Combining the native immunoblot and QconCAT results, the more dominant proteins in the mutant core are ClpR3, ClpP5, ClpP6, ClpP1, and ClpP4, with underrepresentation of ClpP3 (null), ClpP4, ClpR1, ClpR2, and ClpR4. This suggests that a core is assembled with only a subset of ClpPR proteins. Given that ClpP3 is absent and ClpP4 is not well assembled into the Clp core, as compared with ClpP6 in clpp3-1, this suggests a significantly modified core complex in clpp3-1. Interestingly, when considering both core and individual rings, ClpP5, ClpP6, ClpP1, and ClpR3 each overaccumulated between 2- and 2.5-fold in clpp3-1 as compared with the wild type. For ClpP6, this could be confirmed by immunoblotting of the native gels, which also showed that ClpP4 levels...
increased severalfold. In contrast, accumulation levels of ClpR1, ClpR2, and ClpR4 were unchanged; again for ClpR2, this could be confirmed by immunoblotting of the native gels.

Comparison of the Clp assembly states in clpp3-1 with the knockdown mutant clpr2-1 shows that the two mutants have different core complexes. With lower overall levels of ClpR2 in clpr2-1, ClpP4 and ClpP6 assemble into the 350-kD complex, but not as efficiently as in the wild type. It appears as if all copies of ClpR2 are used to assemble a wild-type-like core, with the excess of other ClpPR proteins accumulating mostly in heptameric rings. In contrast, in clpp3-1, only a subset of the Clp subunits assemble in a core complex with a composition substantially different from the wild type, with the remaining subunits accumulating in heptameric rings. Double rings for ClpP4 and ClpP6 were observed in clpp3-1 and clpr2-1 but not in the wild type. In contrast, ClpR2 assembles in a single ring. This indicates heterogeneity in the P-ring in the mutants. It is unlikely that differential association of ClpT can explain this heterogeneity, because immunoblotting showed ClpT1 only in core complexes and barely in 180- to 200-kD rings. Interestingly, ClpT1 showed an upward shift in SDS-PAGE in the case of clpp3-1 but not in clpr2-1. We do not know what this posttranslational modification represents, but a posttranslational modification search based on MS/MS analysis did not show a particular modification, nor alternative processing or splicing.

Proteomes of ClpR2, ClpR4, and ClpP3 Mutants Show a Consistent Phenotype

A comparative quantitative proteomics analysis of clpp3-1 (this study) was integrated with our previous comparative proteome analyses of clpr2-1 (Zybailov et al., 2009a) and clpr4-1 (Kim et al., 2009) in an effort to (1) determine if there are consistent or unique molecular phenotypes across these different Clp core mutants that may help to explain the function of the Clp protease system and its evolutionary adaptation to the chloroplast, (2) find potential Clp substrates observed as up-regulated proteins that overaccumulated because of their increased lifetime due to reduced Clp protease capacity, and (3) provide a basis for the determination of the chloroplast protease network, through the monitoring of accumulation levels of proteases. Here, we will summarize and discuss our findings in the context of these objectives.

In general, we observed very similar proteome phenotypes between clppr2-1, clpr4-1, and our analysis here of clpp3-1. Due to the much improved workflow for clpp3-1, we were able to identify many more significant changes in clpp3-1 than in the other mutants. The consistent phenotype across these mutants indicates that the activities of each of the ClpPR subunits occur through their contribution to a single ClpPR core complex and not because of the activity of individual subunits per se. Collectively, a clear chloroplast phenotype emerges from these Clp core mutants with nine key characteristics: (1) a strong loss of photosynthetic capacity through a systematic loss of the thylakoid-bound photosynthetic machinery and the Rubisco holocomplex (this is consistent with its pale-green phenotype and delayed growth); (2) strong differential up-regulation of plastoglobular proteins, in particular of module 2 of the plastoglobular coexpression network (Lundquist et al., 2012), indicative of a thylakoid membrane homeostasis problem; (3) up-regulation of a subset of DNA/nucleoid-interacting proteins, most likely involved in DNA/genome quality control; (4) differential effects on RNA metabolism; (5) strong up-regulation of protein translation factors, but not at all of plastid ribosomes; (6) systematic up-regulation of stromal chaperone systems; (7) up-regulation of the chloroplast Sec machinery, suggesting a bottleneck in thylakoid protein insertion; (8) up-regulation of a narrow set of chloroplast proteases; and (9) a limited number of changes in envelope transporters and enzymes involved in primary and secondary metabolism (most can be explained by the loss of ATP/NADPH production). A clear example is the up-regulation of the inner envelope ATP/ADP translocators (NTTs), which import cytosolic ATP into the chloroplast, confirming the reduced ATP-generating capacity in the chloroplast (Reinhold et al., 2007). Finally, the selective decrease in plastid-encoded proteins indicates that there is no systematic defect in plastid gene expression in clpp3-1. Only chloroplast-encoded proteins that are part of the photosynthetic apparatus were down-regulated, likely reflecting a systematic down-regulation of the photosynthetic apparatus, including nucleus-encoded proteins. The mechanism for this selective loss of accumulation of chloroplast-encoded protein is unclear but could either be accelerated turnover of thylakoid proteins or a retrograde signaling specifically resulting in down-regulating the expression of thylakoid proteins. Together, this proteome phenotype suggests that the Clp protease system likely has broad substrate specificity and that its general function is essential for chloroplast biogenesis and cannot be replaced by other proteases. Loss of Clp protease capacity specifically affected a subset of chloroplast proteases, indicative of a controlled protease network. Targeted crosses between Clp mutants and other chloroplast protease mutants are in progress in our laboratory to define this network.

The Search for Substrates

An additional motivation for our comparative quantitative proteomics analysis of clpr2-1, clpr4-1, and clpp3-1 was to find up-regulated proteins that overaccumulated because of their increased lifetime due to reduced Clp protease capacity. Indeed, we identified 95 statistically significant up-regulated proteins, but in many cases, these can be explained as compensatory responses to the loss of photosynthetic capacity as well as the destabilization of protein homeostasis, resulting in increased levels of chloroplast chaperone systems and selected proteases. However, there are a number of proteins...

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for which the up-regulation is not so easily explained through pleiotropic effects, in particular those proteins involved in DNA metabolism and the regulation of plastid gene expression. Therefore, we have shifted efforts to more direct substrate protease interactions, focusing on the putative Clp protease regulators (K. Nishimura, Y. Asakura, G. Friso, J. Kim, S.H. Oh, H. Rutschow, L. Ponnala, and K.J. van Wijk, unpublished data).

Why Is the Clp Machinery Necessary in the Embryo Plastids and Subsequent Seedling Development?

Solving the question of why the ClpPR protease is needed for embryogenesis requires an understanding of the role of the plastid in the embryo during seed development. During seed development, embryo plastids begin to differentiate into green chloroplasts at the torpedo stage and then become colorless during seed ripening, as chloroplasts dedifferentiate to nonphotosynthetic plastids by losing their thylakoids and associated chlorophylls. Upon germination, these dedifferentiated plastids are converted into chloroplasts in the cotyledons (Ciamporova and Pretova, 1980) for review of the significance and biogenesis of plastids in embryogenesis, see Hsu et al. (2010); Bryant et al. (2011).

In the light of the recent analysis of embryogenesis mutants, it is perhaps surprising that the ClpP4/ClpP5 proteins are required at that very early state of embryogenesis; most genes in this category are metabolic enzymes involved, for example, in the biosynthesis of acetyl-CoA, folate, etc., although others are involved in protein import and plastid gene expression (Hsu et al., 2010). The lack of normal chloroplast development in the seeds of ClpR2, ClpR4, and ClpP3 null mutants apparently results in a developmental block at the cotyledon stage upon germination. Once this block is broken, by the addition of either Glc or Suc to the growth medium, cotyledons unfold and true leaves are formed. These CLP alleles do not have white cotyledons, as observed for a subset of plastid mutants, the locus white cotyledon1 (Yamamoto et al., 2000), the cya1 stromal elongation factor G (SCO1; Albrecht et al., 2006; Ruppel and Hangarter, 2007), or the thylakoid protein disulfide isomerase CYO1/SCO2 (Shimada et al., 2007; Albrecht et al., 2008; Tanz et al., 2012; for review, see Pogson and Albrecht, 2011). These SCO mutants are not affected in chloroplast development during embryogenesis in the developing silique. After initial growth on Suc, these SCO mutants can be transferred to soil and continue their life cycle under autotrophic conditions, without obvious defects in the chloroplasts of rosette leaves. A different class of mutants have a white-cotyledon phenotype but require sugars to develop green true leaves, such as plastid type I signal peptidase1 (Shipman and Inoue, 2009; Shipman-Roston et al., 2010; Ruppel et al., 2011). Once green leaves have developed, these mutants can grow autotrophically. In contrast, the Clp protease mutants have strong chloroplast phenotypes even when grown under heterotrophic conditions. Thus, the role of the Clp protease system in plant development and plastid function is clearly distinct from the specific developmental program for plastid development in cotyledons. The main challenge now is to determine direct substrates and substrate recognition mechanisms for the Clp system. Based on the collective data set, we hypothesize that proteins involved in DNA metabolism and the regulation of plastid gene expression may represent Clp substrates (e.g., pTAC17). These candidates provide an excellent starting point to study Clp substrate selection.

MATERIALS AND METHODS

Plant Growth, Mutant Isolation, and RT-PCR Analysis

The T-DNA insertion lines in Arabidopsis (Arabidopsis thaliana) Columbia-0 for CLP P3 (AT1G66670) and CLP P4 (AT5g45390) are SALK_000913 and SALK_065330, respectively. The locations of the T-DNA insertions were confirmed by DNA sequencing. Genotyping and RNA extraction were carried out as described previously (Rudella et al., 2006). Various growth conditions are detailed in the figure legends. For RT-PCR, total RNA was isolated with the RNeasy Plant Mini Kit (Qiagen). The first strand was synthesized from equal amounts of total RNA with SuperScript III Reverse Transcriptase (Invitrogen). We tested 15, 20, 25, and 30 cycles for the primer pairs. Fifteen cycles were insufficient to visualize all transcripts, while 20 and 25 cycles best allowed us to visualize the transcripts, and we observed good linearity for 20 and 25 cycles. Primers for genomic PCR and RT-PCR analysis and various complements are listed in Supplemental Table S6.

Complementation

Full-length CLPP4, CLPP5, and CLPP6 cDNA fragments and a 3.424-bp genomic CLPP3 DNA fragment were amplified using Taq polymerase. Primers for complementation are also listed in Supplemental Table S6. The PCR products were subcloned into pCR8/GW/TOPO vector (Invitrogen). Using Gateway LR clonase (Invitrogen), the DNA was introduced into pMDC123 (for genomic), pEARLEYGATE100, or pMDC32 (for cDNA) Gateway destination plant binary vector (Curtis and Grossniklaus, 2003). Agrobacterium tumefaciens transformation, plant transformation, and selection were carried out as described previously (Rudella et al., 2006).

Microscopy of Developing Seeds

Seeds were removed from siliques and cleared for 1 to 24 h in Hoyer’s solution (3.75 g of gum arabic, 50 g of chloral hydrate, and 2.5 mL of glycerol in 15 mL of water) on a microscope slide. Seeds of later developmental stages required extended clearing periods. Cleared seeds were examined using Nomarski optics on an Olympus BX51 microscope.

Plant Growth to Test the Effects of Sugars

To test the sugar effects, the wild type, clpR2-1, and clpR3-1 were grown on one-half-strength Murashige and Skoog agar (0.8% agar) plates with 0%, 1%, 3%, and 5% Suc or Glc under 8-h/16-h light/dark cycles at 40 μmol photons m⁻² s⁻¹.

Phytochemistry

Chlorophyll and carotenoid contents on a fresh weight basis were measured in 80% acetone as described (Lichtenthaler, 1987).

Chloroplast Stroma and Total Leaf Proteome Isolation for Analysis of Clp Assembly States

For chloroplast stroma isolation, leaves of the wild type and various mutant alleles were briefly homogenized in grinding medium (50 msi HEPES-KOH, 5% sucrose, 5% acetone) and centrifuged at 20,000 rpm/100,000 g for 15 min. The chloroplast pellets were resuspended in grinding medium and centrifuged again. The supernatant was used as the chloroplast stroma fraction. The chloroplast stroma was homogenized in grinding medium (50 mM HEPES-KOH, 5% sucrose, 5% acetone) and centrifuged at 20,000 rpm/100,000 g for 15 min. The chloroplast pellets were resuspended in grinding medium and centrifuged again. The supernatant was used as the total leaf protein fraction. The anti-Clp protease antibodies were used to detect Clp protease assembly states in the chloroplast stroma and total leaf protein fractions.
were extracted by grinding 180 mg of fresh leaves in liquid nitrogen into a 5 M EDTA, 5 mM tributylphosphine, and 2.5 mg mL−1 antigens as bait. Additional antisera used were generous gifts from various Escherichia coli (71), ClpP4 (ΔclpP3-1), clpP2-1, and wild-type samples were run on Bio-Rad Criterion Tris-HCl precast gels (10.5%–14% acrylamide gradient). Each of the gel lanes were cut into 20 bands followed by reduction, alkylation, and in-gel digestion with trypsin as described (Shevchenko et al., 2006; Friso et al., 2011). The resuspended peptide extracts were analyzed by data-dependent MS/MS using an online LC-ITQ-Orbitrap (Thermo Scientific). Peptide samples were automatically loaded on a guard column (LC Packings MCL-30-C18(5) via an autosampler followed by separation on a PepMap C18, reverse-phase nanocolumn (LC Packings; nan75-15-03-C18P5) using 90-min gradients with 95% water, 5% acetonitrile, 0.1% formic acid (solvent A) and 95% acetonitrile, 5% water, 0.1% fatty acid (solvent B) at a flow rate of 200 nL min−1. Two blanks were run after every sample (for the gradient and sample injection scheme, see Zybailov et al., 2009b). The acquisition cycle consisted of a survey MS scan in the Orbitrap with a set mass range from 350 to 1,800 m/z-to-charge ratio at the highest resolving power (100,000) followed by five data-dependent MS/MS scans acquired in the LTQ. Dynamic exclusion was used with the following parameters: exclusion size, 500; repeat count, two; repeat duration, 30 s; exclusion time, 180 s; exclusion window, ±6 ppm or ±100 ppm. Target values were set at 5 × 106 and 106 for the survey and tandem MS scans, respectively. The MS survey scan in the Orbitrap was acquired in one microscan. Fragment ion spectra were acquired in the LTQ as an average of three microscans. Protein functions were assigned using the MapMan bin system (Thimm et al., 2004) with accession numbers 16524 to 16643. Significance Analysis of Large-Scale Spectral Counting-Based Quantification The GLEE software was developed in MATLAB version 7 (MathWorks), and a stand-alone executable version of the software code using the MATLAB Compiler was created (A. Poliakov, L. Ponnala, P.D. Olimares, and K.J. van Wijk, unpublished data). GLEE was run in a Windows platform with a cubic polynomial equation fitting, adaptive binning, and 20,000 iterations for the estimation of variation. QSpec analysis was performed in a LINUX platform using the software provided by Kim Choi et al. (2008). A total of 5,000 Markov chain Monte Carlo simulations were performed with 20,000 iterations to ensure convergence of the algorithm. No normalization by protein length or peptide length was included. Assignment of Functional Categories Protein functions were assigned using the MapMan bin system (Thimm et al., 2004) that we further curated and incorporated into the Plant Proteome Database at http://ppdb.tc.cornell.edu/). The MASIC software (Monroe et al., 2008; http://www.pnl.gov/) was used to extract MS and MS/MS relevant statistics such as duty cycle from Thermo raw files. The files for the 120 LC-MS runs were deposited at the Proteomics Identification Database (http://www.ebi.ac.uk/pride/; Vizcaino et al., 2013) with accession numbers 16524 to 16643.

Large-Scale Quantitative Proteomics

Wild-type (Columbia-0) plants were grown on soil for 40 d under a short-day cycle (10 h/14 h of light/dark) at 100 μmol photons m−2 s−1. Homozygous clpP3-1 plants were first grown on agar plates with one-half-strength Mura- shige and Skoog medium and 2% Suc under short-day conditions at 40 μmol photons m−2 s−1, then transferred to soil after 70 d and grown under a short-day cycle at 100 μmol photons m−2 s−1 for another 40 d. Total leaf proteins were extracted by grinding 180 mg of fresh leaves in liquid nitrogen into a fine powder. One milliliter of extraction buffer (1% SDS, 125 mM Tris-HCl [pH 8.8], 5 mM EDTA, 5 mM tributylphosphine, and 2.5 mg mL−1 protease inhibitor Pefabloc) was added, and a pestle was used to solubilize the material. Unsolubilized materials were removed by centrifugation, and proteins in the resulting supernatant were precipitated in 75% acetone at −80°C. Proteins were collected as pellets by centrifugation, followed by two additional acetone washes to remove lipids. The resulting protein pellet was solubilized in 1% SDS and 50 μl Tri-HCl (pH 8.25), and protein concentrations were deter- mined using the BCA Protein Assay Kit (Thermo Scientific). Fifty micrograms total leaf protein of clpP3-1 and wild-type samples was run on Bio-Rad Criterion Tris-HCl precast gels (10.5%–14% acrylamide gradient). Each of the gel lanes were cut into 20 bands followed by reduction, alkylation, and in-gel digestion with trypsin as described (Shevchenko et al., 2006; Friso et al., 2011). The resuspended peptide extracts were analyzed by data-dependent MS/MS using an online LC-ITQ-Orbitrap (Thermo Scientific). Peptide samples were automatically loaded on a guard column (LC Packings MCL-30-C18(5) via an autosampler followed by separation on a PepMap C18, reverse-phase nanocolumn (LC Packings; nan75-15-03-C18P5) using 90-min gradients with 95% water, 5% acetonitrile, 0.1% formic acid (solvent A) and 95% acetonitrile, 5% water, 0.1% fatty acid (solvent B) at a flow rate of 200 nL min−1. Two blanks were run after every sample (for the gradient and sample injection scheme, see Zybailov et al., 2009b). The acquisition cycle consisted of a survey MS scan in the Orbitrap with a set mass range from 350 to 1,800 m/z-to-charge ratio at the highest resolving power (100,000) followed by five data-dependent MS/MS scans acquired in the LTQ. Dynamic exclusion was used with the following parameters: exclusion size, 500; repeat count, two; repeat duration, 30 s; exclusion time, 180 s; exclusion window, ±6 ppm or ±100 ppm. Target values were set at 5 × 106 and 106 for the survey and tandem MS scans, respectively. The MS survey scan in the Orbitrap was acquired in one microscan. Fragment ion spectra were acquired in the LTQ as an average of three microscans. Protein data processing, data searching against The Arabidopsis Information Resource 8 using Mascot, and subsequent filtering and quantification based on normalized and adjusted spectral counts were carried out as described (Zybailov et al., 2009b) and as outlined in Supplemental Figure S2. MS-derived information, as well as annotation of protein name, location, and function for the identified proteins, can be found in the Plant Proteome Database (http://ppdb.tc.cornell.edu/). The MASIC software (Monroe et al., 2008; http://www.pnl.gov/) was used to extract MS and MS/MS relevant statistics such as duty cycle from Thermo raw files. The files for the 120 LC-MS runs were deposited at the Proteomics Identification Database (http://www.ebi.ac.uk/pride/; Vizcaino et al., 2013) with accession numbers 16524 to 16643.

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