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Identification of new protein substrates for the chloroplast ATP-dependent Clp protease supports its constitutive role in *Arabidopsis*

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Short title: Multiple new substrates for chloroplast Clp protease in *Arabidopsis*

Abbreviations used: Chl, chlorophyll; EF, elongation factor; FW, fresh weight; FBP ALD, fructose biphosphate aldolase; Hsp, heat shock protein; Lox2, lipoxygenase 2, NDP, nucleoside diphosphate ; PPIase, peptidyl-prolyl *cis-trans* isomerase; Rubisco, ribulose-1,5-bisphosphatecarboxylase/oxygenase; SSU, small subunit of Rubisco; UPRT, uracil phosphoribosyl-transferase;

SYNOPSIS

The ATP-dependent Clp protease in plant chloroplasts consists of a heterogeneous proteolytic core containing multiple ClpP and ClpR paralogs. In this study, we have examined in detail the only viable knockout mutant to date of one of these subunits in *Arabidopsis thaliana*, ClpR1. Loss of ClpR1 caused a slow-growth phenotype, with chlorotic leaves during early development that later partially recovered upon maturity. Analysis of the Clp proteolytic core in the *clpR1* mutant (*clpR1-1*) revealed ca. 10% of the wild type levels remaining, likely due to a relative increase in the closely related ClpR3 protein and its partial substitution of ClpR1 in the core complex. A proteomic approach using an *in organello* proteolytic assay revealed 19 new potential substrates for the chloroplast Clp protease. Many of these substrates were constitutive enzymes involved in different metabolic pathways including photosynthetic carbon fixation, nitrogen metabolism and chlorophyll/heme biosynthesis, whereas others function in housekeeping roles such as RNA maturation, protein synthesis and maturation, and recycling processes. In contrast, degradation of the stress-related chloroplast proteins Hsp21 and lipxygenase 2 were unaffected in the *clpR1-1* line and thus not facilitated by the Clp protease. Overall, we show that the chloroplast Clp protease is principally a constitutive enzyme that degrades numerous stromal proteins; a feature that almost certainly underlies its vital importance for chloroplast function and plant viability.

Key words: *Arabidopsis thaliana*, chloroplast, proteolysis, stroma.

INTRODUCTION

Molecular chaperones and proteases are integral components of the quality control processes in the actively changing cellular environment of all living organisms. Chaperones comprise a large group of proteins with diverse functions and are involved in such processes as protein folding/unfolding and in complex assembly/disassembly [1]. Additionally, some chaperones interact with proteases and are responsible for recognizing polypeptide substrates and unfolding them prior to degradation [2]. Proteases have an equally important role and are responsible for degrading damaged proteins that might otherwise accumulate to potentially harmful levels or begin to impair associated processes. Besides these housekeeping activities, proteases are also involved in recycling amino acids and regulating the stability of key enzymes and regulatory proteins (reviewed in [3]).

Plant chloroplasts are dynamic organelles which, consistent with their endosymbiotic origin, contain various proteases of bacterial ancestry. Of these chloroplast proteases, the best characterized to date are FtsH proteases anchored to the stromal surface of the thylakoid membranes, Deg proteases bound extrinsically to both sides of the thylakoid membrane, and the stromal-localized Clp protease (reviewed in [4]). The Clp protease is an ATP-dependent serine-type protease present in eubacteria, plants and mammals [5]. This two-component enzyme, composed of one Hsp100 chaperone partner complexed to a proteolytic core, is best described in *Escherichia coli*. The central proteolytic core of the *E. coli* Clp protease is comprised of two apposing heptameric rings of ClpP with the proteolytic active sites sequestered within the internal cavity [6]. This barrel-shaped core is flanked on one or both ends by a single homogeneous hexameric ring of an Hsp100 partner, either ClpA or -X [7]. The Hsp100 partner recognizes, unfolds and translocates the protein substrate through the narrow entrance of the Clp proteolytic core into the central chamber in an energy-dependent manner [8-10]. Substrates are then rapidly degraded into small peptides that eventually diffuse out of the core complex [11].

Higher plants have by far the greatest diversity of Clp proteins, with at least 23 different types in the model species *Arabidopsis thaliana* [5,12]. Of these, nine are Hsp100 chaperones (ClpB1-3, ClpC1-2, ClpD, and ClpX1-3), six are paralogs of the proteolytic subunit ClpP (ClpP1-6), and four are paralogs of a ClpP-like variant (ClpR1-4) which lacks the Ser-type catalytic triad and has recently been shown to be proteolytically inactive in cyanobacteria (Andersson et al., submitted). In addition, *Arabidopsis* has an ortholog to the bacterial adaptor protein ClpS as well as two unique Clp proteins with sequence similarity to the N-terminal domain of Hsp100 proteins, which here are renamed ClpT1-2 (from ClpS1-2) to avoid confusion with the functionally distinct ClpS adaptor. The bulk of Clp proteins in *Arabidopsis* are located in the chloroplast stroma (ClpB3, ClpC1-2, ClpD, ClpP1, ClpP3-6, ClpR1-4, ClpS and ClpT1-2), with all except the plastomic ClpP1 encoded in the nucleus [13-15]. Despite the numerous Clp proteins residing in the stroma, only one Clp proteolytic core has been identified [14,16]. This 325-350 kD complex consists of two sub-complexes, presumably representing two heptameric rings by analogy to the ClpP core complexes in other organisms. One ring consists of ClpP1 and ClpR1-4, while the other contains ClpP3-6 [17]. In addition, a single subunit each of ClpT1 and ClpT2 associates peripherally to the proteolytic core [16]. ClpT1 has been further shown to bind to the ClpP3-6 sub-complex [17].

Previous studies using various transgenic plants have demonstrated the crucial role of the chloroplast Clp protease. Reduced amounts of the plastomic ClpP1 or other components of the Clp proteolytic core results in plants impaired in chloroplast differentiation, shoot development and overall plant viability [17-22]. In *clpP6* antisense plants, for example, the amount of the chloroplast Clp proteolytic core decreased by 80-90%, consistent with the level of antisense repression of the ClpP6 subunit. These antisense lines were also used in the identification of the first few putative protein substrates for the chloroplast Clp protease [17]. In contrast, no viable *Arabidopsis* mutants for any of the nuclear-encoded ClpP or -R paralogs have been reported to date, despite extensive screening of all available T-DNA insertion lines [5]. The one exception to this was an EMS mutant, *clpR1-1*, the characterization of which has so far been concentrated on five-day old seedlings [22]. In this study, we have now characterized in detail the *clpR1-1* mutant at a later stage of development. The absence of

ClpR1 leads to a chlorotic, slow growing phenotype as previously observed [22], and plants with reduced photosynthetic rates. Despite the loss of ClpR1, a small amount of intact Clp proteolytic core is formed in the *clpR1-1* line, likely due to partial compensation by the ClpR3 subunit. Moreover, we have now used the *clpR1-1* line to identify many new potential protein substrates for the stromal Clp protease; some of which are involved in general homeostatic roles while others in more specific metabolic pathways. We also reveal that certain stress-inducible stromal proteins are not degraded by the Clp protease, emphasizing its role principally as an essential constitutive housekeeping enzyme.

EXPERIMENTAL

Plant growth conditions

Arabidopsis thaliana wild type (ecotype Columbia-0) and *clpR1-1* mutant [22] were grown in soil containing 20% (v/v) perlite in controlled environment chambers. Seeds were vernalized at 4°C for a minimum of 48 h to break dormancy. All plants were grown individually in pots or as lawns under the following standard conditions: 8 h photoperiod with white light (ca. 150 $\mu\text{mol m}^{-2} \text{s}^{-1}$), 23/18°C day/night temperatures, and 65% RH. These conditions were found to improve the growth of the *clpR1-1* line from those used previously, in which seeds were first germinated on ½ MS medium containing 1% (w/v) sucrose, grown under 12h photoperiod for the first 10 d, then transferred to soil and placed in 16 h photoperiod [22]. Unless stated otherwise, all experiments were performed with wild type *Arabidopsis* and *clpR1-1* plants at the same developmental age (5 and 7 weeks old, respectively).

Chlorophyll and photosynthetic measurements

Chlorophyll (Chl) was extracted from leaf discs or small leaves and measured as described previously [23]. F_v/F_m and ETR parameters were measured on leaves using a pulse-amplitude modulated fluorometer (PAM-2000; Heinz-Waltz, Effeltrich, Germany) also as described earlier [23]. Statistical significance was determined using the *t*-test in which a *p* value < 0.01 was significantly different.

Protein complex separation by native-PAGE

Intact chloroplasts were isolated from wild type and *clpR1-1* leaves as described previously [17,24]. Protein concentration in each sample was determined using the BCA protein assay according to the manufacturer's protocol (Pierce). The Tris/borate-based PAGE system [25] was then used to separate native soluble protein complexes (60 μg protein) from wild type and *clpR1-1*. Samples were electrophoresed to ensure proteins reached their pore size limitation within the gel matrix as previously described [26]. Molecular mass standards were ferritin (440 kD monomer, 880 kD dimer), urease (272 kD trimer), and BSA (66 kD monomer, 132 kD dimer). After native-PAGE, proteins were transferred to nitrocellulose and the Clp protein complexes were detected using specific polyclonal antibodies as previously described [15,17,23]. Primary antibodies were detected with the horseradish peroxidase-linked anti-rabbit IgG secondary antibody from donkey (Amersham Pharmacia) and visualized by enhanced chemiluminescence (ECL Advance; GE Healthcare) using the ChemiGenius² imaging system (Syngene).

2D-PAGE

Stromal protein fractions were obtained from isolated chloroplasts [17] and purified further using the ReadyPrep 2D Cleanup Kit (Bio-Rad). Purified protein (450 μg) was resuspended in isoelectric focusing (IEF) buffer (8 M urea, 2 M thiourea, 50 mM DTT, 4% CHAPS, 0.2% (v/v) Bio-Lyte pH 3-10 ampholytes, 0.0002% (w/v) bromophenol blue) and loaded on 24 cm IEF ReadyStrips (IPG nonlinear pH 4 to 7; Bio-Rad). Strips were rehydrated for 12 h at 20°C in 24 cm strip holders, followed by IEF on an Ettan IPGphor using the standard separation program. After focusing, strips were immediately equilibrated for SDS-PAGE in DTT buffer (6 M urea, 2.5% (v/v) SDS, 20% (v/v) glycerol, 2% (w/v) DTT, 0.002% (w/v) bromophenol blue, 50 mM Tris-HCl pH 8.8) for 15 min followed by a 10 min incubation in iodoacetamide buffer (6 M urea, 2.5% (v/v) SDS, 20% (v/v) glycerol, 2.5% (w/v) iodoacetamide, 0.002% (w/v)

bromophenol blue, 50 mM Tris-HCl pH 8.8). IEF strips were then placed on 12% (v/v) acrylamide SDS-PAGE gels (Tris-Gly) and run overnight at 20°C in a Ettan DALT II system (GE Healthcare). Gels were electrophoresed in simultaneous sets of four to reduce variation, with triplicate gels run for each treatment. Gels were stained with Flamingo™ fluorescent stain (Bio-Rad), scanned, and spots were then analyzed using specialized software ImageMaster™ 2D Platinum 5.0 (GE Healthcare). Spot volumes were normalized to the total spot volume on each gel. Spots were selected for mass spectrometric identification based on significant differences in the three replicates between sample pairs; identification was done with MALDI-TOF MS or HPLC-MS/MS (Finnigan LTQ-FT; Thermo Electron) using the software Mascot (at Matrix Science, U.K.) at the SweGene Proteomics Center (Göteborg University).

Protein degradation assay

Intact chloroplasts from the lawns of wild type and *clpR1-1* plants at the same developmental age (3 and 5 weeks, respectively) were isolated and counted, as previously described [17]. An equal number of intact chloroplasts were incubated for 0 and 3 h in 60 $\mu\text{mol m}^{-2} \text{s}^{-1}$ light at 25°C in the presence of an ATP regeneration system [17]. Samples were then ruptured in 5 volumes of rupture buffer (10mM MgCl_2 , 20mM HEPES-NaOH pH 7.6) and frozen in liquid nitrogen. Thawed samples were centrifuged at 20,000g for 10 min, after which the stromal supernatants were transferred to new tubes. Protein concentration was determined using the BCA protein assay described above. For proteins >60 kD 1D-PAGE (precast 3 to 8% polyacrylamide Tris-acetate gels; Invitrogen) was used, followed by staining with Coomassie Brilliant Blue G 250. For proteins <60 kD 2D-PAGE (described in detail above) was used. All proteins identified as potential substrates for the Clp protease were quantified. Proteins exhibiting >25% degradation in wild type chloroplasts during the time course of the assay were identified by MALDI-TOF MS and HPLC-MS/MS (SweGene Proteomics Center, Göteborg University).

For the Hsp21 degradation assay, fully developed wild type and *clpR1-1* plants grown under standard conditions were heat stressed under conditions similar to that described previously [27]. Plants were first preheated at 40°C for 30 min 4h into the light period. After 20.5h recovery, the plants were again heat shocked at the start of the next light period by gradually raising the growth temperature to 40°C during the first h, maintaining 40°C for a further 4h, then gradually lowering the temperature to 25°C during the following h. During all high temperature treatments, the light was kept at an irradiance of 350 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Ca. 3-d after the heat shock treatment (i.e., 65 h), intact chloroplasts were isolated in three independent batches each containing chloroplasts from three individual plants as described [17]. For the degradation assays, intact chloroplasts (1.5×10^5 chloroplasts / μl) were incubated at 25°C under 80 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ for 0, 1.5 and 3 h. At each time point, an equal number of chloroplasts (4.5×10^5) were solubilized in NuPAGE sample buffer (Invitrogen) and separated by denaturing PAGE as previously described [17]. Immunoblotting was performed as detailed above using antibodies specific for Hsp21.

RESULTS

Loss of ClpR1 causes leaf chlorosis and impaired photosynthesis

In an earlier study, we identified an *Arabidopsis* EMS mutant lacking all ClpR1 protein, termed *clpR1-1* [22]. However, since only five-day old seedlings with chlorotic cotyledons were used for most of this previous work, we undertook a more comprehensive analysis of *clpR1-1* in more mature plants. The growth conditions were also changed from that used previously [22] to optimise the growth of the *clpR1-1* line relative to the wild type. As shown in Figure 1A, the *clpR1-1* line had a slow growing, chlorotic phenotype. Chlorosis was most severe in younger inner leaves and as the leaves expanded the severity of chlorosis gradually lessened. The growth in *clpR1-1* was delayed by ca. 2 weeks relative to the wild type. Due to this, all comparisons between wild type *Arabidopsis* and *clpR1-1* were performed on plants of the same developmental size rather than age.

The amount of Chl in *clpR1-1* compared to wild type was analyzed in both outer and inner leaf whorls, given the difference in severity of chlorosis. In wild type plants the Chl

content of older outer leaves was significantly lower (1.59 ± 0.03 nmol mg fresh weight (FW) $^{-1}$; $n=3$) than in younger inner leaves (2.72 ± 0.03 nmol mg FW $^{-1}$; $n=3$) (Figure 1B). The opposite was true in *clpR1-1*, with the younger inner leaves having much less Chl (0.59 ± 0.04 nmol mg FW $^{-1}$; $n=3$) compared to the outer leaves (1.1 ± 0.04 nmol mg FW $^{-1}$; $n=3$), consistent with their more severe chlorotic appearance. Despite the partial recovery in the older outer leaves of *clpR1-1*, the Chl content remained significantly lower than in the outer wild type leaves.

We next compared different photosynthetic parameters in the *clpR1-1* leaves relative to those of the wild type. Measuring first the photochemical efficiency of photosystem II (PSII) (F_v/F_m) revealed no significant differences between inner (0.839 ± 0.001 ; $n=3$) and outer (0.837 ± 0.002 ; $n=3$) leaves in the wild type (Figure 1C). In contrast, the chlorotic inner leaves of *clpR1-1* showed a marked inhibition of PSII photochemical efficiency (0.723 ± 0.006 ; $n=3$). Although this recovered somewhat in the older outer leaves (0.758 ± 0.011 ; $n=3$), the photochemical efficiency remained less than that in wild type outer leaves. Photosynthetic electron transport rates also showed a decrease in both photosynthetic efficiency (i.e., quantum yield as determined by the initial slope of the light-response curve) and capacity (as measured at the highest irradiance) in both the inner and outer leaves of *clpR1-1* (Figure 1D). Again, the younger inner leaves were more drastically affected, correlating with the partial recovery of the phenotype in more developed *clpR1-1* plants.

Reduced levels of photosynthetic proteins in *clpR1* mutant

Due to the reduced photosynthetic performance and Chl content in the *clpR1* mutant, we next analyzed the relative amounts of photosynthetic protein complexes using antibodies specific to the following marker proteins: small subunit (SSU) of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco); Lhcb2 and D1 for the PSII outer antennae and reaction center, respectively; PsaL for PSI and β -subunit of ATPase (Figure 2). The levels of all marker proteins decreased significantly (40-55%) in the inner leaves of *clpR1-1* relative to the wild type, consistent with the chlorotic phenotype and reduced Chl levels. In comparison, no significant change in Rubisco, PSI, PSII reaction center or ATPase contents were observed in the outer leaves of *clpR1-1* relative to the wild type, whereas a small decrease (ca. 15%) was observed in the levels of PSII outer antennae (results not shown). Interestingly, a larger molecular mass form of Lhcb2 accumulated in the *clpR1* mutant in both inner (Figure 2) and outer leaves (results not shown). This had been observed previously [22] as well as in a *clpR2* knock-down mutant in which it was demonstrated to be an unprocessed precursor of Lhcb2 containing the chloroplast transit peptide [20]. When summed together the abundance of Lhcb2 was only 20% reduced in the inner leaves of *clpR1-1* and unchanged in the outer leaves.

Reduced levels of Clp proteolytic core in *clpR1* mutant

The plastidic Clp proteolytic core contains multiple ClpP and ClpR paralogs in addition to ClpT1 and -T2 in a ca. 335 kD complex [16]. The core is composed of two distinct sub-complexes, one containing ClpP1 and ClpR1-4, and the other containing ClpP3-6 [17]. Given that all subunits of the proteolytic core other than ClpR1 appear to be essential for plant viability, we next investigated how the loss of ClpR1 affected the formation of the core complex. Stromal proteins isolated from both wild type and *clpR1-1* plants were separated by native-PAGE and then used for immunoblotting to detect the intact Clp proteolytic core (335 kD) and the ClpP1, R1-4 sub-complex (230 kD) (Figure 3A, Supplementary Figure). Immunoblotting confirmed that even though ClpR1 was totally absent in the mutant line a small amount of the Clp proteolytic core remained ($9.9\% \pm 4.7\%$ [$n=23$] as quantified from the ClpP1, R2, R4, P3-6 and T1 antibodies). Analysis of the ClpP1/R1-4 sub-complex revealed a two-fold decrease in the ClpP1, -R2 and -R4 proteins in the *clpR1* mutant, but little change to ClpR3 content (Figure 3A). This infers that ClpR3 is most likely able to partially compensate for the lack of ClpR1 forming some stable ClpP1/ClpR2-4 sub-complex and thereby the residual amount of Clp proteolytic core.

In addition to the ClpP1, ClpR1-4 sub-complex, the ClpP3-6 sub-complexes with (200 kD) or without ClpT1 (180 kD) were also examined for possible changes resulting from the

loss of ClpR1. As shown in Figure 3B, there was a significant shift in the proportion of both ClpP3-6 sub-complexes in *clpR1-1* relative to the wild type. There was a 2-3 fold increase in the larger 200 kD complex containing ClpT1 in *clpR1-1* while the smaller 180 kD sub-complex decreased by 35-50%. Overall, there was a marginal increase in the combined amount of ClpP3-6 sub-complexes (on average 25%) in *clpR1-1* compared to the wild type. Despite this, the amount of intact Clp proteolytic core complex (335 kD) detected in the *clpR1-1* mutant using the various ClpP3-6, ClpT1 antibodies was again only ca. 10% of the wild type level (Figure 3B), indicating that the slightly higher amount of the ClpP3-6 sub-complex could not compensate for the loss of ClpR1. Moreover, no ClpP3-6 protein was detected in the 230 kD ClpP1, ClpR1-4 sub-complex, again indicating that neither of the ClpP3-6 proteins could substitute for ClpR1 within the Clp proteolytic core complex.

Identification of new protein substrates for the chloroplast Clp protease

Recent research on *clpP6* antisense lines revealed the first six putative substrates for the chloroplast Clp protease in higher plants [17]. Given that these substrates were all involved in various constitutive metabolic and homeostatic functions, we first tested if the Clp protease also targeted chloroplast polypeptides associated with certain stress responses. The first protein examined was lipoxygenase 2 (Lox2), which is required for the wound-induced accumulation of jasmonic acid in *Arabidopsis* [28]. Since Lox2 is constitutively expressed in addition to being stress inducible, we simply examined the degradation rate of Lox2 in chloroplasts isolated from non-stressed leaves. Comparing wild type and *clpR1-1* chloroplasts, however, revealed no significant difference in the degradation of Lox2 during the 3 h time course (Figure 4). We next examined an exclusively stress-inducible protein that is not synthesized under normal growth conditions; the major small HSP localized in chloroplasts of higher plants (Hsp21) during heat stress. It is well known that following its induction and accumulation in heat stressed leaves, stromal Hsp21 is steadily degraded during the recovery period at lower temperatures [27,29]. To determine if the Clp protease was responsible for this degradation of Hsp21, we isolated intact chloroplasts from both wild type *Arabidopsis* and *clpR1-1* plants at the early stages of this recovery phase. As shown in Figure 4, however, no significant difference was observed in the degradation rate of Hsp21 in wild type and *clpR1-1* chloroplasts.

Given the lack of degradation of the stress-related proteins Lox2 and Hsp21 by the chloroplast Clp protease, we next pursued a more comprehensive search for additional constitutive protein substrates. For this, a similar approach to that described previously [17] was used, except that the *clpR1-1* line was used instead of the *clpP6* antisense lines since it has less of the Clp proteolytic core remaining and a more stable phenotype among individual plants. The methodology employed was to first identify significant changes in stromal protein content in *clpR1-1* relative to the wild type. This was based on the rationale that substrates should accumulate when the levels of Clp protease are considerably reduced. One-dimensional (1D) denaturing-PAGE gels were used for proteins >70 kD (data not shown) and two-dimensional (2D) PAGE was used to resolve smaller proteins, <70 kD. Ten proteins accumulated more than 3-fold in the *clpR1* mutant (Figure 5, Table 1). Of these, all were detected in the wild type except for a putative ribulose 5-phosphate isomerase (protein number 6). The identity of the remaining nine proteins were three other Calvin cycle enzymes, fructose biphosphate aldolase (FBP ALD) and two paralogs of Rubisco small subunit (SSU), SSU 1A and SSU 2B; as well as two RNA-binding proteins (cp29), a uracil phosphoribosyl-transferase (UPRT), a cyclophilin peptidyl-prolyl *cis-trans* isomerase (PPIase), a nucleoside diphosphate (NDP) kinase, and a putative fibrillin. Five of these proteins (i.e., ribulose 5-phosphate isomerase, RNA-binding protein, cp29, UPRT, PPIase and NDP kinase) were previously identified as proteins that also accumulated in the *clpP6* antisense lines [17].

An *in organello* chloroplast degradation assay was then used to compare the stability of different stromal proteins in wild type *Arabidopsis* and the *clpR1* mutant. Both 1D- and 2D-PAGE were again used to resolve the degradation of stromal proteins over a 3 h time course. The amount of protein at time 0 and 3 h were quantified for both wild type and *clpR1-1* based on staining with either coomassie blue (1D-PAGE) or Flamingo™ (2D-PAGE). A slower

degradation rate in *clpR1-1* was observed for three large proteins: elongation factor containing Ts motifs (EF-Ts), the molecular chaperone Hsp90, and a RNA helicase (Figure 6A, Table 2). After 3 h the amount of each of these proteins decreased in wild type by 25-45%, whereas in *clpR1-1* no significant change was observed from the time zero levels (Figure 7A). Each of these proteins were also more abundant in *clpR1-1* relative to wild type, albeit by less than 2-fold. These three proteins were all identified earlier as substrates in the *clpP6* antisense plants [17]. In addition, 22 small molecular mass substrates were also identified (Figure 6B, Table 2), including eight of the proteins that accumulated in *clpR1-1* (Table 1); the other two proteins, SSU1A and SSU2B (spots no. 9 and 10), did not decrease in the wild type during the 3 h degradation assay and were therefore not considered potential substrates. As shown in Figure 7A, all the putative substrates significantly decreased (25-80% loss) in the wild type throughout the 3 h degradation assay. In the case of one substrate (RNA-binding protein, spot no. 2), the protein could not be detected in wild type chloroplasts after 3 h, indicating most if not all of it was degraded during the assay time course. Most of these identified proteins (16 of the 22) were very stable in *clpR1-1* and their levels did not significantly change during the 3 h time course. No degradation rate could be determined for PPlase (protein number 6) in the wild type, as it was not detected at either time point with the fluorescent stain used. However, since PPlase was earlier identified as a putative substrate in the wild type using the more sensitive silver staining [17], its lack of degradation in the *clpR1* mutant confirmed this original finding and therefore it was included as a substrate in this study. For the six remaining putative substrates a slight decrease in the amount of each protein was observed in *clpR1-1*, ranging from 10-20%. Of the 25 substrate proteins identified, all but six were also more abundant in *clpR1-1* (Table 2).

Relative abundance of identified substrates in developing leaves

Since the chlorotic phenotype of the *clpR1* mutant was more severe in younger leaves relative to mature ones, we next analyzed the relative amount of each newly identified protein substrate in young (inner) and mature (outer) leaves of the wild type. It should be noted that the corresponding analysis for substrates no. 6-8 and 11-13 have already been shown [17]. As shown in Figure 7B, around half of the new protein substrates (spot no. 3, 14, 16, 20, 21, 23-25) showed no significant variation in amount between the younger inner and mature outer leaves. Most of these unchanged polypeptides were enzymes within the different metabolic processes, whereas some such as EF-G and Hsp70-6 were involved in protein synthesis and maturation. Of the remaining protein substrates, four (no. 19, 22, 26, 27) were comparatively more abundant in wild type outer leaves, with again a mixture of metabolic enzymes and general housekeeping proteins. In contrast, five protein substrates (no. 2, 4, 15, 17, 18) were significantly less abundant in the outer leaves, all of which are involved in homeostasis or structural functions within the chloroplast. For the two remaining putative substrates (no. 1, 5), no data could be obtained since both were relatively low abundant and could not be reliably detected in the replicate experiments. Altogether, when combining the new substrates to those identified previously [17], more than a third of the total number of substrates (9 of 25) was more abundant in the younger inner leaves, correlating to the more severe chlorotic phenotype in these tissues.

DISCUSSION

In this study, we have detailed the phenotypic effects resulting from the loss of ClpR1 in *Arabidopsis*, as well as identified many more possible protein substrates for the chloroplast Clp protease. The *clpR1-1* line is apparently the only viable knockout mutant for any of the chloroplast-localized ClpP and ClpR proteins. The phenotype of *clpR1-1* was very similar to that previously observed using *clpP4* and *clpP6* antisense lines [17,21] and a *clpR2* knock-down T-DNA insertion line [20]. Plants were chlorotic, with the younger inner leaves more affected than the older outer leaves. This chlorosis in young leaves affected their rate of development, being around 2 weeks behind those of the wild type. The overall phenotype of *clpR1-1* was, unsurprisingly, more severe than that of the *clpP6* antisense lines [17] since all the ClpR1 protein was lost in the mutant line. Although leaf chlorosis lessened with age in

clpR1-1 the outer leaves never completely greened, whereas the outer leaves of *clpP6* antisense lines fully recovered to become indistinguishable from the wild type. The resulting delay in growth was also ca. 1 week longer in *clpR1-1* relative to the *clpP6* antisense lines. The *clpR1-1* line had significantly lower Chl content and impaired photosynthetic activity in both younger inner and older outer leaves, again consistent with the somewhat more severe phenotype of this mutant compared to the *clpP6* antisense lines, in which significant changes only occurred in inner leaves relative to wild type [17]. The amounts of the major photosynthetic protein complexes were also reduced by 40-50% in the chlorotic inner leaves of *clpR1-1*, again more severe than that in the corresponding leaves of the *clpP6* antisense lines (i.e., 30-40% reduction). These features suggest that although the Clp protease obviously plays a more critical role in chloroplasts of younger developing leaves, it still performs an important function in more mature leaves.

The model Clp proteolytic core in *E. coli* is composed of 14 identical ClpP subunits arranged in two face-to-face heptameric rings [6]. In the chloroplast stroma of higher plants a single heterogeneous Clp proteolytic core exists, consisting of five ClpP and four ClpR paralogs [14]. Additionally, this oligomer contains two novel proteins, ClpT1 and ClpT2 that associate peripherally to the main ClpP/R complex, forming a proteolytic core of 325-350 kD [16]. The Clp proteolytic core in *Arabidopsis* chloroplasts consists of two sub-complexes, presumably heptameric rings, one containing ClpP1 and ClpR1-4 and the other with ClpP3-6 which can also associate to ClpT1 [17]. Interestingly, loss of ClpR1 greatly reduced the amount of the core complex (by 90%) but did not completely abolish it. Indeed, the 230 kD sub-complex normally containing ClpR1 had less than half the wild type levels of ClpP1, ClpR2 and ClpR4 in *clpR1-1* but unchanged amounts of ClpR3. This relative increase of ClpR3 inferred that it was partially compensating for the absent ClpR1 within the 230 kD sub-complex. This modified sub-complex must then associate, albeit with much lower affinity to the ClpP3-6 sub-complex, forming sufficient amounts of functional, or partially functional, Clp proteolytic core (i.e., 10%) to enable the *clpR1-1* line to remain viable. The possibility that ClpR3 can partially substitute for ClpR1 function is supported by the fact that ClpR3 has the greatest sequence similarity to ClpR1 relative to the other subunits of the Clp proteolytic core [14]. The partial replacement of ClpR1 with ClpR3 in the *clpR1-1* line is the only evidence to date for any degree of redundancy regarding the stoichiometry and oligomerization of the Clp proteolytic core. Mutant lines for any of the other subunits of the proteolytic core complex have so far proven lethal, suggesting they are each essential for the formation of an active chloroplast Clp protease.

Interestingly, loss of ClpR1 caused a slight increase in the amount of the ClpP3-6 sub-complex, with a significantly greater proportion of the larger sub-complex containing ClpT1. This is in contrast to when the level of the ClpP3-6 sub-complex is greatly reduced as in *Arabidopsis clpP6* antisense lines, with the ClpP1, ClpR1-4 sub-complex also decreasing but to a lesser extent (i.e., by 25%; [17]). The accumulation of the larger ClpP3-6, ClpT1 sub-complex in *clpR1-1* suggests this is an intermediate assembly step prior to the association of the two different sub-complexes (i.e., ClpP1, ClpR1-4 and ClpP3-6) into the intact Clp proteolytic core complex, although the mechanistic details of how this process occurs remains unclear. Overall, it appears that the fully active form of the chloroplast Clp proteolytic core requires the correct stoichiometry of each ClpP and ClpR subunit, which recently has been proposed for each sub-complex as 1:(3):1:2 for ClpP1, (-R1 + -R3), -R2, -R4; and 1:3:2:1 for ClpP3-6 [17].

One of the more exciting advances in the field of the ATP-dependent Clp protease in chloroplasts has been the development of an *in organello* proteolytic assay that can be used for identifying potential native protein substrates [17]. Until recently the only known substrate for chloroplastic Clp proteases was the cytochrome *b₆/f* complex identified in the green alga *Chlamydomonas reinhardtii* during nitrogen starvation [30]. Using this assay to compare wild type *Arabidopsis* with *clpP6* antisense lines, six substrates were identified for the stromal Clp protease. The function of all six proteins was generally related to chloroplast homeostasis and not to any specific metabolic pathways, supporting the hypothesis that the Clp protease has a major 'housekeeping' role. In this study, we have expanded upon this early work to now

identify many more putative protein substrates for the chloroplast Clp protease. Up to 25 soluble proteins were found as potential substrates, including the six that were earlier identified (i.e., EF-Ts, Hsp90, RNA helicase, UPRT, PPIase, and NDP kinase; [17]). Most of the identified proteins accumulated in the *clpR1* mutant relative to the wild type, as would be expected for a substrate whose degradation was impaired. Of the 25 substrate proteins, most (ca. 60%) were related to general housekeeping activities, again consistent with the Clp protease being primarily involved in chloroplast homeostasis. Most of the housekeeping representatives were molecular chaperones. Hsp90 was one of the original substrates identified, and is involved in regulating the activity of key regulatory proteins in addition to cooperating with other chaperone systems, such as Hsp70 and PPIase in the correct folding of nascent polypeptides [31]. Interestingly, as with Hsp90, PPIase was also identified as a substrate in the previous search and we have now found two Hsp70 proteins, Hsp70-7 and Hsp70-6. In addition, the two subunits of the chloroplast chaperonin 60 (Cpn60 α/β) were identified as substrates, which together are essential for the correct folding, and assembly when necessary of many soluble proteins in the chloroplast [32].

Several components of chloroplast protein synthesis were also identified as putative substrates of the Clp protease. EF-Ts, another of the initial substrates found [17] acts as a nucleotide exchange factor for the EF-Tu. EF-Tu itself, along with the homologous elongation factor G (EF-G) also now appear to be substrates. EF-Tu mediates the entry of the aminoacyl tRNA into a free site of the ribosome, then EF-Ts catalyzes the release of GDP from EF-Tu, enabling EF-Tu to bind to a new GTP molecule and go on to catalyze another aminoacyl tRNA addition. EF-G then catalyzes the translocation of the tRNA and mRNA down the ribosome at the end of each round of polypeptide elongation [33]. Participation of these elongation factors significantly accelerates the elongation of nascent polypeptides by ribosomes [34], and as such their degradation by the Clp protease might play a key regulatory role in the process of chloroplast protein synthesis. Proteins involved in RNA maturation were also identified as possible Clp proteolytic substrates. RNA-binding proteins play a major role in regulating mRNA metabolism in chloroplasts and three types are found in *Arabidopsis* (cp29, cp31 and cp33; [35]). Here we identified two RNA-binding proteins (both cp29 types) that accumulated in *clpR1-1*, both of which turned out to also be substrates. Moreover, RNA helicase 3 (another of the original substrates; [17]), was also found as a Clp proteolytic substrate in *Arabidopsis*. RNA helicases are essential for many homeostatic processes such as transcription, mRNA processing, initiation of translation and ribosome biogenesis [36]. Given their functions, it is possible that impaired degradation of these RNA-related substrates might be related to the delay in chloroplast ribosomal RNA maturation previously observed in the *clpR1-1* line [22]. The final two substrates involved in 'housekeeping' activities were UPRT and NDP kinase, proteins also previously identified as substrates [17]. UPRT is involved in salvaging uracil for recycling to allow for continued synthesis of pyrimidine nucleotides [37], whereas NDP kinase helps regulate the available nucleotide pool by generating nucleoside triphosphates [38].

Although many of the proteins identified in this study as putative substrates for the chloroplast Clp protease perform in general housekeeping functions, almost half (ca. 40%) had more specific cellular functions. Five substrates were enzymes of the photosynthetic Calvin cycle (fructose biphosphate aldolase, ribose 5-phosphate isomerase, phosphoribulokinase, phosphoglycerate kinase, and triose-phosphate isomerase), while two were enzymes involved in nitrogen metabolism (glutamine synthetase 2 [GS2] and a regulatory protein of GS2 termed uridylyltransferase [UTase]). GS2 plays an essential role in nitrogen metabolism by catalyzing the condensation of glutamate and ammonia to form glutamine. Because of its critical role, GS2 is an enzyme that is tightly regulated by a complex signal transduction pathway. The internal cellular ammonium levels are first detected by UTase, which in turn alters the uridylation state of PII proteins. These proteins occupy a pivotal position in the network regulating the activity of GS [39,40]. Another substrate of the Clp protease was an enzyme of the Chl and heme biosynthetic pathway, coproporphyrinogen III oxidase (coprogen oxidase). Coprogen oxidase catalyzes the oxidative decarboxylation of coproporphyrinogen III (Coprogen) to proto-porphyrinogen IX. Coprogen is a very photosensitive tetrapyrrole and regulation of this enzyme guarantees a constant flux of

metabolic intermediates and avoids photodynamic damage by accumulating porphyrins [41]. Indeed, it is possible that accumulation of coprogen oxidase in *clpR1-1* might result in accumulation of the downstream product Mg-protoporphyrin IX (Mg-protolX). Accumulation of Mg-ProtolX inhibits the accumulation of both nuclear- and plastid-encoded proteins in chloroplasts [42,43] and this might be one explanation for the previously reported low amounts of mRNA encoding for plastid destined proteins in *clpR1-1* [22]. The three remaining protein substrates were the antioxidant 2-Cys Prx A, a putative fibrillin and a hypothetical protein predicted to be a nucleoside diphosphate sugar epimerase. Peroxiredoxins are a large group of proteins that participate in cell proliferation, differentiation, apoptosis, and photosynthesis. 2-Cys Prx A regulates the concentration of hydrogen peroxide to protect against oxidative damage as well as regulates peroxide-mediated signal transduction pathways [44]. In plastids of plants, fibrillins are thought to be involved in maintaining the structural stability of plant lipid bodies [45], and are believed to play a role in plant responses to environmental stress [46]. Epimerases or 'dehydratases' are enzymes that regulate the stereochemistry of carbohydrates. Carbohydrates play important roles in photo-assimilation as well as serve as structural constituents of galactolipids and glycoproteins and transport metabolites. Therefore epimerases are involved in many metabolic pathways [47].

Of the various protein substrates now identified for the chloroplast Clp protease, less than half were more abundant in younger leaves of wild type plants compared to mature leaves. Of those that were, however, almost all are proteins intricately involved in general housekeeping roles such as protein synthesis, folding and maturation, as well as RNA maturation. Greater amounts of these regulatory proteins is almost certainly related to more metabolically active chloroplasts in younger leaves, with relatively high rates of transcription, translation and protein turnover. As such, therefore, it is consistent that the near absence of the Clp protease due to repression or inactivation of one of the constituent subunits causes more severe phenotypic changes in younger leaves as observed in this study and others [20-22]. The function of these protein substrates in such housekeeping duties is also consistent with the Clp protease being present in plastids other than chloroplasts, including those in the non-photosynthetic parasitic plant *Epifagus* [48] and Apicomplexan protozoan [49]. Interestingly, although the Clp protease is also present in *Arabidopsis* roots [16], its relative content is considerably lower than that in leaves [15]. The greater abundance of Clp protease in leaves might not only be related to higher plastid numbers per cell compared to roots, but it might also be due to many of the protein substrates identified in this study functioning in chloroplast-specific processes such as photosynthesis.

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FIGURE LEGENDS

Figure 1. Loss of ClpR1 produces a chlorotic phenotype, and reduces Chl content and photosynthetic performance

(A) Phenotypic comparison of plants at the same developmental stage: 5 and 7 week old wild type (Wt) *Arabidopsis* and *clpR1-1*, respectively. Plants were grown under the same standard conditions of 23/18°C day/night temperatures, 8 h photoperiod with 150 $\mu\text{mol m}^{-2} \text{s}^{-1}$ light, and 65% RH. Chl content (B) and photochemical efficiency of PSII (F_v/F_m) (C) in outer and inner leaves of Wt and *clpR1-1*. Values shown are averages \pm SE ($n=3$). (D) Photosynthetic electron transport (ETR) rates in outer and inner leaves of Wt and *clpR1-1*. ETR rates were measured at different PAR levels between 0 and 400 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Values shown are from three independent Wt and *clpR1-1* plants. In panels B-D, plants were compared at the same developmental stage as shown in (A).

Figure 2. Changes in levels of photosynthetic proteins in the *clpR1* mutant.

(A) Amounts of marker proteins for different photosynthetic protein complexes in inner leaves of 5 week wild type *Arabidopsis* and 7 week *clpR1-1* were determined by immunoblotting. Total cell extracts were isolated and separated based on equal protein content by 1D-PAGE. Antibodies were used to detect specific marker proteins for each photosynthetic protein complexes: SSU for Rubisco; Lhcb2 and D1 for PSII; PsaL for PSI; and β -subunit for ATPase. (B) Quantification of the amount of each photosynthetic marker protein in the inner leaves of *clpR1-1* relative to wild type. Values shown are averages \pm SE ($n=3$) with the wild type values set at 100%.

Figure 3. Clp proteolytic core complexes in Wt *Arabidopsis* and *clpR1* mutant

Clp proteolytic core complexes from 3 week Wt *Arabidopsis* and 5 week *clpR1-1* were separated by native-PAGE on the basis of equal protein content. (A) The intact Clp proteolytic core complex (335 kD) and the sub-complex containing ClpP1 and ClpR1-4 (230 kD) were visualized by immunoblotting using specific antibodies as indicated below each panel. The graph shows the quantification of the amount of the 230 kD Clp proteolytic core sub-complex in *clpR1-1* relative to Wt. Values shown are averages \pm SE ($n=3$), with the Wt values set at 100%. (B) The intact Clp proteolytic core complex (335 kD) and the sub-complexes containing ClpP3-6 (180 kD) or ClpP3-6, ClpT1 (200 kD) were visualized by immunoblotting using specific antibodies as indicated below each panel. The graph shows the quantification of the amount of the 180 and 200 kD Clp sub-complexes in *clpR1-1* relative to Wt. Values shown are averages \pm SE ($n=3$), with the Wt values set at 100%.

Figure 4. Chloroplast Clp protease does not degrade certain stress-related polypeptides

(A) Equal amount of intact chloroplasts from Wt and *clpR1-1* plants were incubated 0-3 h in the presence of light and ATP. For Lox2, plants were grown under standard growth conditions. For Hsp21, plants were first heat shocked at 40°C as described in the Methods and then allowed to recover for 65 h under the standard growth conditions. Isolated chloroplast proteins from different time points were separated by denaturing-PAGE and the Hsp21 and Lox2 proteins visualized by immunoblotting using specific antibodies. (B) Degradation of Hsp21 and Lox2 in Wt and *clpR1-1* chloroplasts. Values shown are averages \pm SE ($n=3$), where protein content from the 0 h sample was set to 100%.

Figure 5. Changes in stromal protein composition in Wt *Arabidopsis* and *clpR1-1* line

Stromal proteins isolated from 3 week Wt *Arabidopsis* and 5 week *clpR1-1* plants were separated by 2D-PAGE and visualized by Flamingo™ staining. Proteins consistently more abundant in the *clpR1-1* relative to the Wt are circled and numbered. Shown are representative results from three replicates. The identity of each numbered protein is detailed in Table 1.

Figure 6. Identification of protein substrates for the chloroplast Clp protease

Intact chloroplasts from 3 week Wt *Arabidopsis* and 5 week *clpR1-1* were incubated for 3 h in the presence of ATP and light. Chloroplasts were then ruptured and fractionated. Stromal proteins were separated by SDS-PAGE and visualized by coomassie blue staining **(A)** or by 2D-PAGE and visualized by Flamingo™ **(B)**. Those proteins whose abundance significantly decreased over the 3 h time course in Wt but not in *clpR1-1* were identified by MALDI-TOF MS. Shown are representative gels from three independent degradation assay replicates. **(A)** Arrows at the right indicate the three putative protein substrates. **(B)** Circled and numbered spots indicate 23 putative protein substrates. Where appropriate the number corresponds to that in Figure 5. The identity of each numbered protein is detailed in Table 2.

Figure 7. Putative protein substrates of the chloroplast Clp protease and their abundance during development

(A) Extent of degradation for 25 putative protein substrates of the Clp protease in intact chloroplasts from Wt *Arabidopsis* and *clpR1-1* over the 3 h time course. Stromal proteins were separated by 1D- or 2D-PAGE and quantified after visualization by coomassie blue or Flamingo™, respectively. The relative amount of protein remaining after 3 h is shown as an average \pm SE ($n=3$) plotted as a percent of the time 0 value, which was set to 100%. Each number corresponds to a numbered protein in Figure 5, the identity of which is detailed in Table 2. *Indicates protein that accumulated in *clpR1-1* but was not detected in the Wt at T=0 or at T=3. It has previously been identified as a protein substrate using *clpP6* antisense lines [17]. **(B)** Stromal proteins from inner and outer leaves of 8 week Wt *Arabidopsis* were separated by 2D-PAGE and quantified by Flamingo™. The relative amounts of each newly-identified substrate are shown as an average \pm SE ($n=3$) plotted as a percent of the inner leaf value, set to 100%. Each number corresponds to a numbered protein in Figure 6, which is further detailed in Table 2.

Table 1

Table 1. Identification of stromal proteins accumulating in <i>clpR1-1</i> line						
Spot No.	Identity	Mass (kD) ¹	Protein Identified ²	Protein Score ³	Peptides Matched ⁴	Fold ⁵
1	Fructose biphosphate aldolase	42	At2g21330	817	14	6
2	Putative RNA binding protein (cp29)	33	At3g53460	571	11	4
3	Putative RNA binding protein (cp29)	31	At2g37220	460	8	4
4	Putative fibrillin	30	At4g04020	316	5	3.5
5	Putative ribose 5-phosphate isomerase	28	At3g04790	534	11	5
6	Peptidyl-prolyl <i>cis-trans</i> isomerase	28	At3g62030	581	12	∞
7	Uracil phosphoribosyl-transferase	22	At3g53900	432	18	10
8	Nucleoside diphosphate kinase	19	At5g63310	182	8	5
9	Rubisco small subunit 2B	14	At5g38420	134	8	13
10	Rubisco small subunit 1A	14	At1g67090	119	8	15

Identification by MALDI-TOF MS and HPLC-MS/MS of those proteins most abundant in the *clpR1* mutant relative to the wild type. Each numbered protein corresponds to the same numbered protein circled in Figure 4.

¹ Molecular mass calculated from gel size markers.

² Protein identification as *Arabidopsis* Genome Initiative gene code.

³ Protein scores > 66 are significant (P < 0.05).

⁴ Peptide match at mass tolerance of 6100 ppm, allowing a maximum of one missed cleavage.

⁵ Fold upregulation based on quantification of stained gel spots.

Table 2

Table 2. Identity of putative protein substrates for the chloroplast Clp protease as determined by MALDI-TOF MS and HPLC-MS/MS

Spot No.	Identity	Mass (kD) ¹	Protein Identified ²	Protein Score ³	Peptides Matched ⁴	Fold ⁵
1	Fructose biphosphate aldolase	42	At2g21330	817	14	6
2	RNA binding protein	34	At3g53460	571	11	4
3	Putative RNA binding protein (cp29)	31	At2g37220	460	8	4
4	Putative fibrillin	30	At4g04020	316	5	3.5
5	Putative ribose 5-phosphate isomerase	28	At3g04790	534	11	5
6*	Peptidyl-prolyl <i>cis-trans</i> isomerase	28	At3g62030	581	12	∞
7	Uracil phosphoribosyl-transferase	22	At3g53900	432	18	10
8	Nucleoside diphosphate kinase	19	At5g63310	182	8	5
11	Elongation factor Ts	150	At4g29060	222	14	1.5
12	Hsp90-5	87	At2g04030	184	19	1.6
13	RNA helicase 3	85	At5g26742	178	16	1.8
14	Elongation factor G	79	At1g62750	176	17	1.6
15	Hsp70-7	70	At5g49910	100	11	1.7
16	Hsp70-6	70	At4g24280	103	11	1.6
17	Chaperonin 60α	59	At2g28000	198	17	2
18	Chaperonin 60β	59	At1g55490	98	11	1.7
19	Elongation factor Tu	50	At4g20360	108	12	1.7
20	Phosphoglycerate kinase	48	At3g12780	129	11	1
21	Glutamine synthetase 2	46	At5g35630	808	16	1
22	Phosphoribulose kinase	42	At1g32060	132	11	1
23	Coproporphyrinogen III oxidase	41	At1g03475	660	15	1
24	Putative uridylyltransferase	36	At1g16880	470	10	1.2
25	Hypothetical protein	35	At2g37660	663	14	1
26	Triose phosphate isomerase	32	At2g21170	716	12	1
27	2-cys peroxiredoxin A	27	At3g11630	476	8	1.4

Each numbered protein corresponds to the same numbered protein circled in Figure 5.

¹ Molecular mass calculated from gel size markers.

² Protein identification as *Arabidopsis* Genome Initiative gene code.

³ Protein scores > 66 are significant (P < 0.05).

⁴ Peptide match at mass tolerance of 6100 ppm, allowing a maximum of one missed cleavage.

⁵ Fold upregulation based on quantification of stained gel spots.

* Not detected in wild type. Previously identified as a substrate by Sjögren et al., (2006).

Figure 1

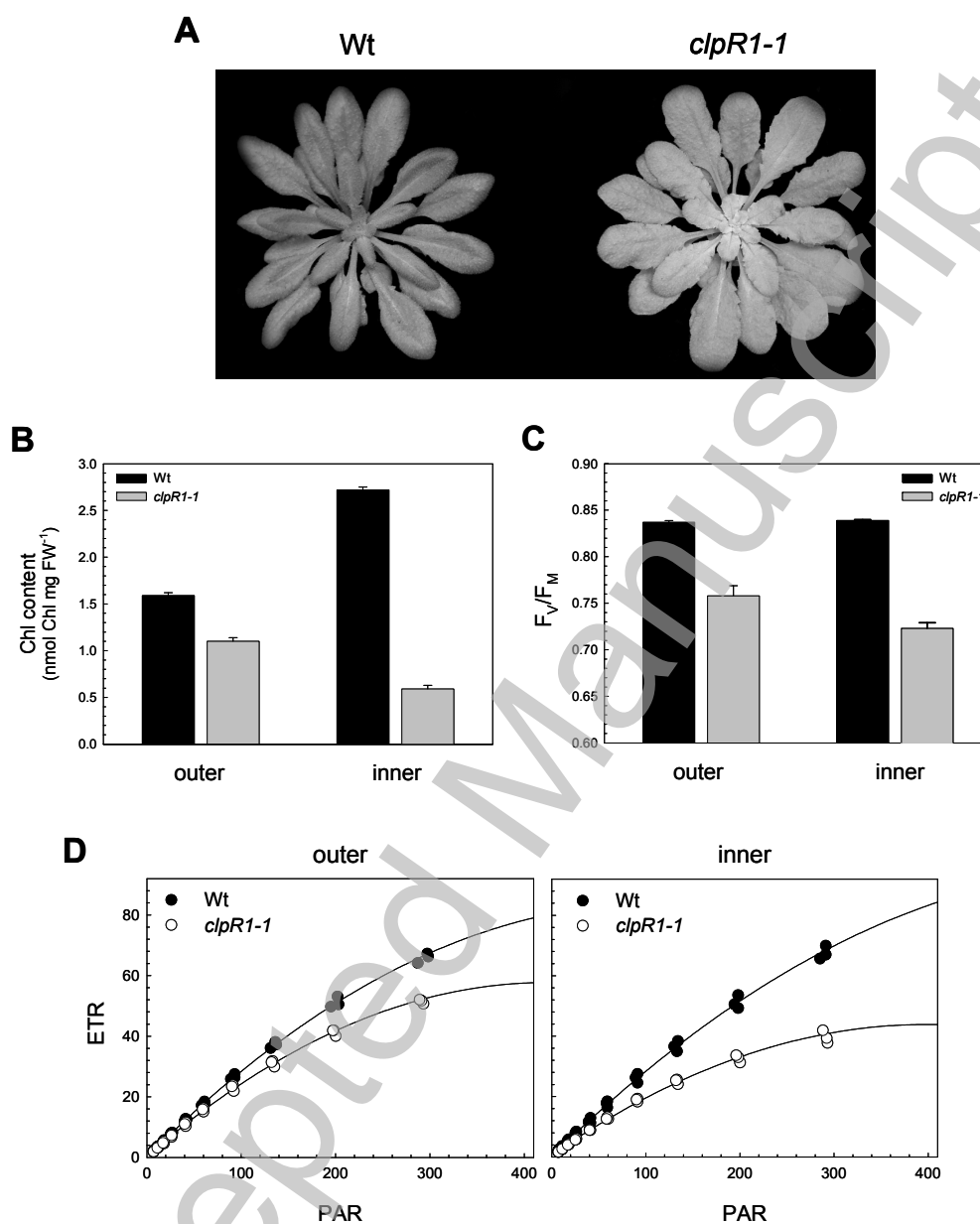


Figure 2

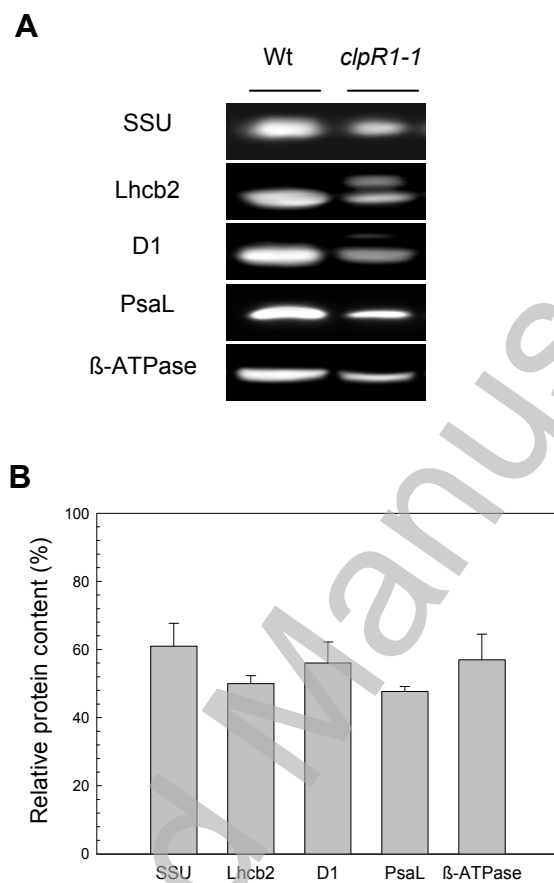


Figure 3

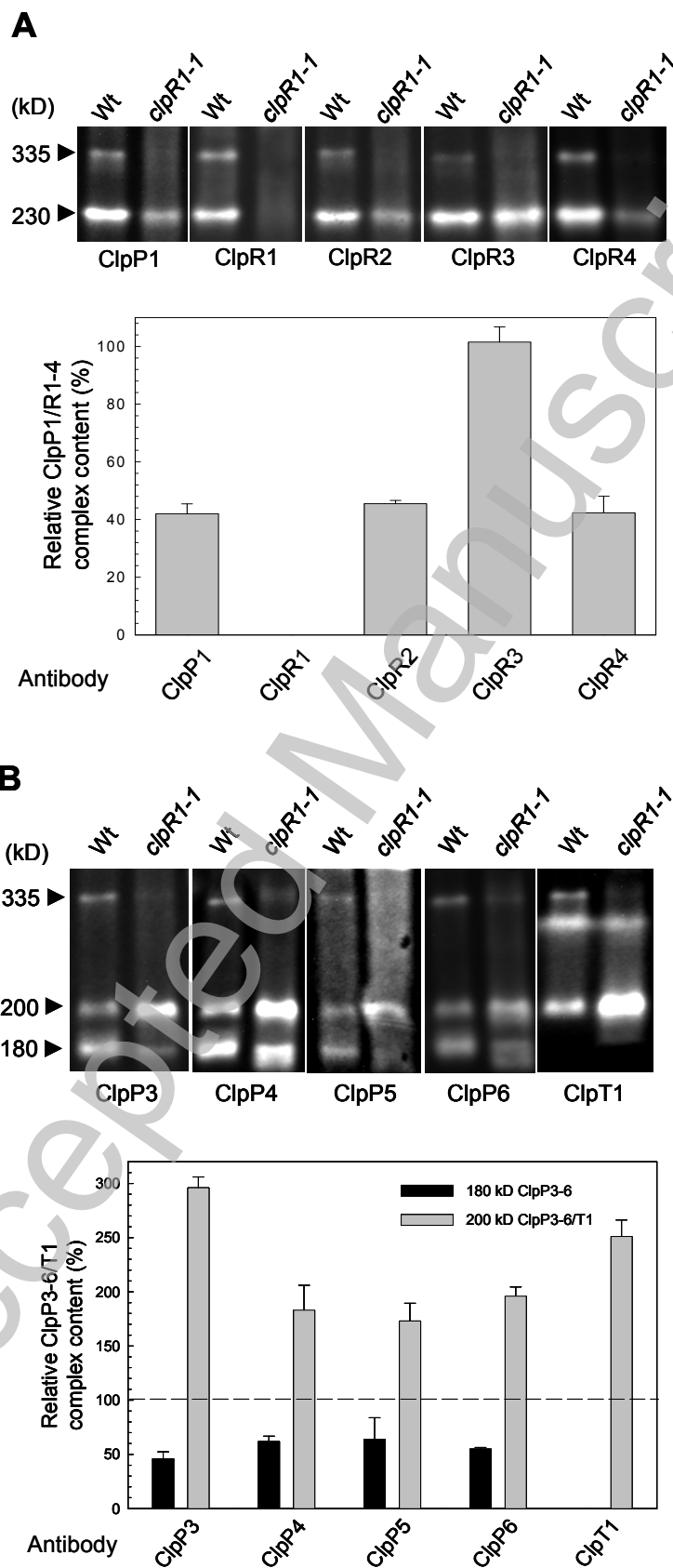


Figure 4

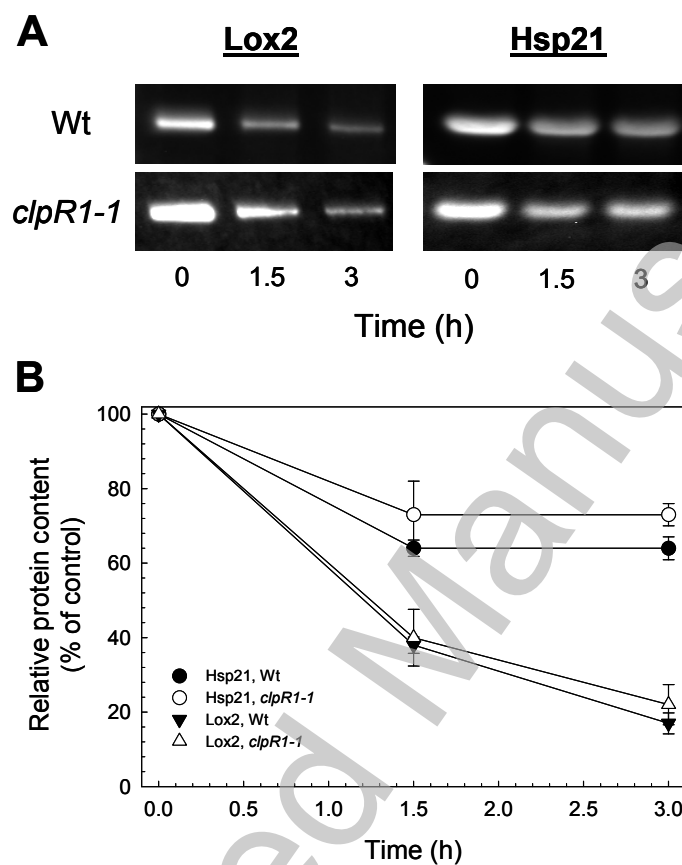


Figure 5

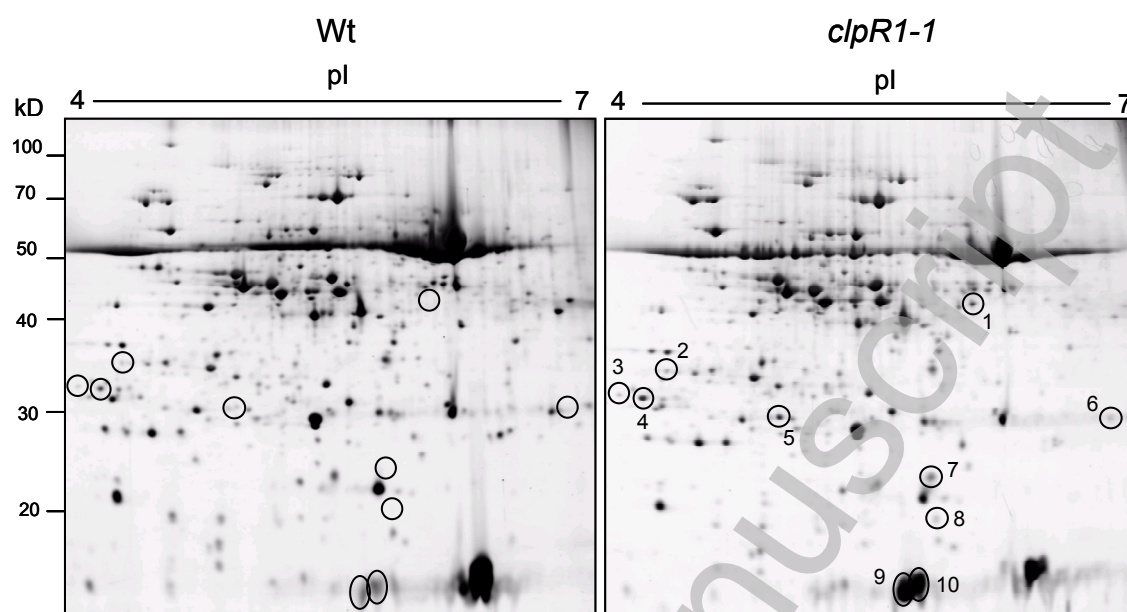


Figure 6

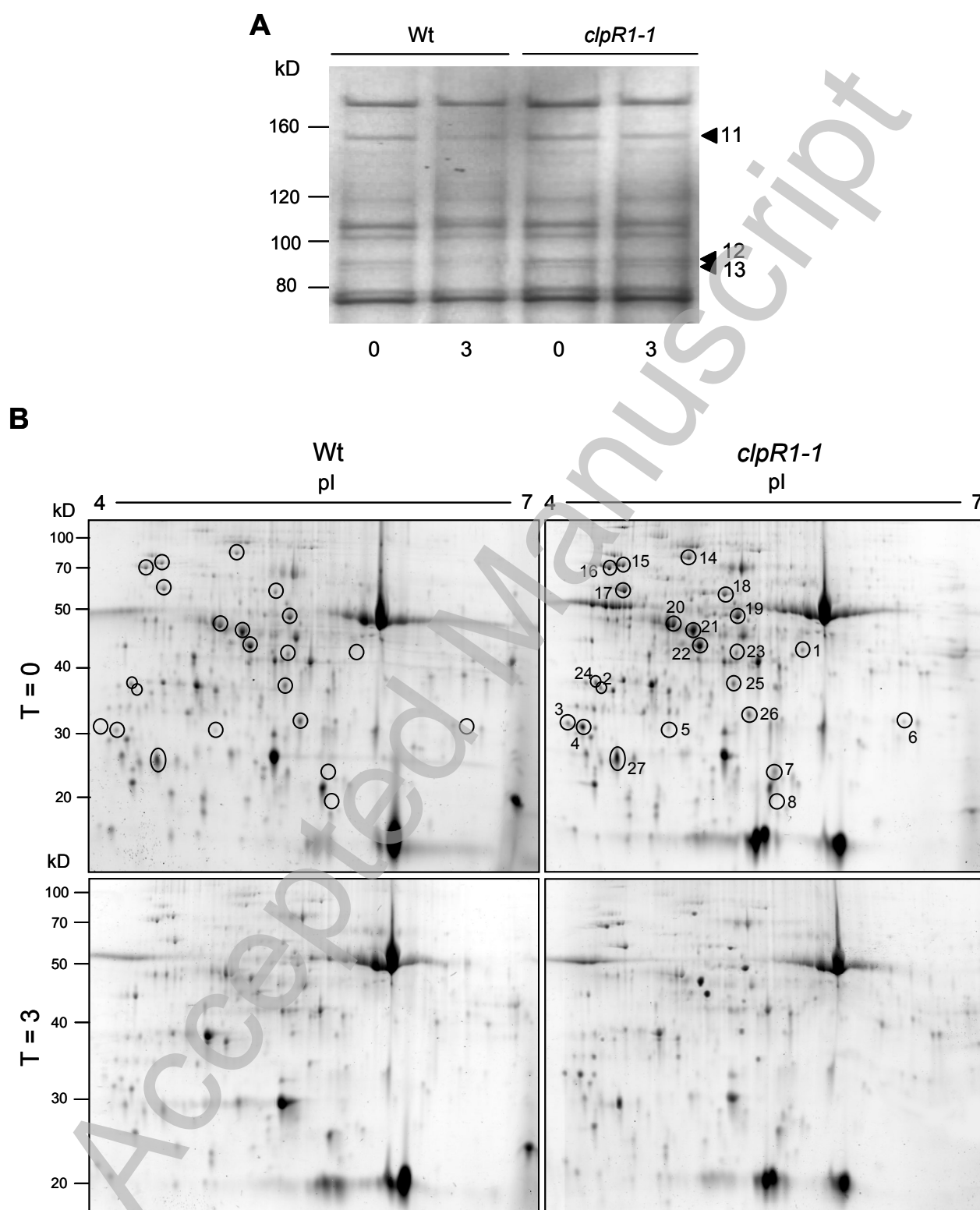


Figure 7

