

Characterization of Chloroplast Clp proteins in *Arabidopsis*: Localization, tissue specificity and stress responses

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The ATP-dependent Clp protease is one of the newly identified proteolytic systems in plant organelles that incorporate the activity of molecular chaperones to target specific polypeptide substrates and avoid inadvertent degradation of others. We describe new nuclear-encoded ClpC (ClpC1) and ClpP (ClpP3–5) isomers in *Arabidopsis thaliana* that raise the total number of identified Clp proteins to 19. The extra Clp proteins are localized within the stroma of chloroplasts along with the ClpD, –P1 and –P6 proteins. Potential differential regulation among these Clp proteins was analysed at both the mRNA and protein level. A comparison between different tissues showed increasing amounts of all plastid Clp proteins from roots to stems to leaves suggested the greatest abundance of proteins was in chloroplasts. The increases in protein were mirrored at the mRNA level for most ClpP isomers (ClpP1, –3, –4 and –6) but not for the three Hsp100 proteins (ClpC1, –C2 and –D) and ClpP5,

which exhibited little change in transcript levels, suggesting post-transcriptional/translational regulation. Potential stress induction was also tested for all chloroplast Clp proteins by a series of brief and prolonged stress conditions. Short-term moderate and severe stresses (desiccation, high salt, cold, heat, oxidation, wounding and high light) all failed to elicit significant or rapid increases in any chloroplast Clp protein. However, increases in mRNA and protein content for ClpD and several ClpP isomers did occur during long-term high light and cold acclimation of *Arabidopsis* plants. These results reveal the great complexity of Clp proteins within the stroma of plant chloroplasts, and that these proteins, rather than being rapidly induced stress proteins, are primarily constitutive proteins that may also be involved in plant acclimation to different physiological conditions.

Introduction

The protein environment inside plastids is one of the more dynamic ones in plant cells, and yet much remains unclear about the mechanisms by which these complex protein interactions are regulated. Molecular chaperones are a ubiquitous group of regulatory proteins well known to directly influence the structure and function of many polypeptides. They assist the folding, assembly and translocation of numerous cellular proteins during both normal and adverse growth conditions. Certain chaperones also target irreversibly damaged proteins for proteolysis, thereby preventing the accumulation of potentially cytotoxic polypeptides (Parsell and Lindquist 1993). Integral to this degradative process is the direct interaction between certain molecular chaperones and proteases, many of which are dependent on ATP. In plastids of higher plants, three such chaperone/proteolytic

mechanisms have so far been identified, of which all are homologous to well characterized proteases from eubacteria (Adam et al. 2001). The DegP protease extrinsically attached to the lumenal and stromal surfaces of thylakoid membranes, and the FtsH proteases also located on the stromal surface, both incorporate chaperone and proteolytic activities within the one polypeptide (Lindahl et al. 1996, Itzhaki et al. 1998, Haubühl et al. 2001). The soluble Clp protease, however, has separate chaperone and proteolytic subunits, and like the other two proteolytic systems, little is yet understood about their precise functions in plastids.

Most knowledge about the Clp protease is derived from extensive studies on the model enzyme from *Escherichia coli* (reviewed by Porankiewicz et al. 1999). The ATP-dependent Clp protease is a large complex of two

oligomeric subunits: a serine-type protease (ClpP) and a regulatory ATPase (ClpA or -X). In *E. coli*, the Clp protease has a central core comprised of two face-to-face heptameric rings of ClpP, which are flanked at one or both sides by a single hexameric ring of ClpA or -X (Grimaud et al. 1998). In an ATP-dependent fashion, the regulatory subunits selectively bind and unfold the polypeptide targeted for degradation and then translocate it into the central cavity of the barrel-like ClpP complex (Wang et al. 1997, Singh et al. 2000). Within this proteolytic chamber, ClpP rapidly degrades the polypeptide into smaller fragments that then diffuse out. Because of the different protein specificities of the regulatory subunits, the ClpAP and ClpXP complexes can be considered as distinct proteases.

ClpA and ClpX are members of a new family of molecular chaperones known as Hsp100/Clp. The family can be separated into two broad groups, with proteins in the first (ClpA-E) having two different ATP-binding domains (ATP-1 and ATP-2) while those in the second (ClpX, -Y) have only one such domain (Schirmer et al. 1996). The five types of proteins in the first group are distinguished by conserved amino acid sequences, length of the spacer region separating the ATP-1 and -2 domains, and the organism they originate from. Hsp100 proteins are thought to function with a common mechanism of dismantling oligomeric protein complexes, as has been shown for *E. coli* ClpA and -X (Wickner et al. 1994, Levchenko et al. 1995). When associated with ClpP, certain Hsp100 proteins perform dual regulatory/chaperone activities, thereby influencing the eventual fate of selected protein substrates.

Accompanying the detailed biochemical/structural analysis of Clp proteases in *E. coli* has been an increasing number of studies of Clp proteins in many different bacteria and eukaryotes, and studies of their importance for vital processes such as sporulation, DNA replication, protein turnover, stress tolerance and acclimation, and regulation of gene expression (Porankiewicz et al. 1999). In particular, plants as shown in the model species *Arabidopsis thaliana* possess by far the greatest number of Clp proteins, with isomeric forms of ClpP and most Hsp100 members. Of the Hsp100 proteins, several nuclear-encoded varieties are present in plants, with ClpB proteins located in the cytosol (Schirmer et al. 1994), ClpX in mitochondria (Halperin et al. 2001) and ClpC and -D in chloroplasts (Moore and Keegstra 1993, Weaver et al. 1999). In comparison, six distinct ClpP isomers have now been identified in *Arabidopsis* (ClpP1-6, Adam et al. 2001), all of which are nuclear-encoded except for the plastomic ClpP1 protein. *Arabidopsis* also has four additional genes encoding proteins with overall sequence similarity to ClpP but which all lack the proteolytic active site characteristic of ClpP (i.e. Ser-His-Asp catalytic triad), and as such they have been distinguished as ClpR (Clarke 1999, Adam et al. 2001).

Because of their recent discovery and great complexity, much remains unknown about the regulation, structure and function of Clp proteins in *Arabidopsis*, especially

those localized in chloroplasts. Genetic evidence to date has shown chloroplastic ClpC and ClpP1 are essential constitutive proteins (Shanklin et al. 1995, Shikanai et al. 2001). The chloroplast Clp proteins reported so far (ClpC, -D, -P1 and -P6) are localized in the stroma (Moore and Keegstra 1993, Shanklin et al. 1995, Sokolenko et al. 1998, Weaver et al. 1999), with possible associations between ClpC and ClpP1/P6 (Desimone et al. 1997, Sokolenko et al. 1998). In this study we describe the identification of additional isomeric forms of ClpC and ClpP in *Arabidopsis*, and the localization of these proteins in the chloroplast stroma. We also detail the first concerted approach to determine simultaneously the expression and synthesis characteristics of most chloroplast Clp proteins in various tissues and under a range of physiological conditions.

Materials and methods

Cloning, sequencing and data analysis of *Arabidopsis* clp genes

A computer search of the *Arabidopsis* EST database using known ClpC and ClpP sequences from other plant species was performed to identify cDNA clones for novel *clp* genes in *Arabidopsis*. Partially sequenced clones coding for a single ClpC and four ClpP polypeptides were identified and obtained from Dr Amie Franklin and the *Arabidopsis* Resource Center (Ohio State University, OH, USA), respectively. The most intact cDNA clones for each gene were then completely sequenced using the Taq DyeDeoxy terminator cycle sequencing kit (DYEnamic™, Amersham Pharmacia Biotech, Uppsala, Sweden) and analysed on an automated sequencer (ABI377, Perkin Elmer, Foster City, CA, USA). Sequences were viewed with the AutoAssembler (PE Applied Biosystems, Foster City, CA, USA) computer software and then analysed with the Genetics Computer Group (GCG Wisconsin Package, Accelrys Inc., CA, USA) program. Predictions of intracellular locations and transit peptide processing sites were done using TargetP version 1.01 (Emanuelsson et al. 2000) and ChloroP version 1.01 (Emanuelsson et al. 1999).

Production of specific antibodies

Specific polyclonal antibodies were generated against *Arabidopsis* Clp proteins using either fusion proteins or synthetic peptides. For ClpD, -P1 and -P6, C-terminal fusion proteins with the maltose-binding protein (MBP) were over-expressed in *E. coli* using the inducible pMAL-c2 vector (New England Biolabs, Beverly, MA, USA). For ClpD, the 510 bp 3' region downstream of the ATP-2 domain was PCR amplified with the high fidelity *pfu* DNA polymerase (Stratagene, La Jolla, CA, USA). The *clpD* fragment was ligated in-frame to the 3'-end of the *maltE* gene coding for MBP in the pMAL-c2 plasmid and transformed into *E. coli* DH5 α . Similar fusion constructs with MBP were made for the 5' exon of *clpP1*

and the entire *clpP6* gene. Over-expression of all fusion constructs from the *tac* promoter was induced by addition of IPTG to exponentially growing cells. Fusion proteins were then purified according to the method by Riggs (1990). For the remaining ClpP isomers, specific antibodies were made to KLH-or BSA-conjugated synthetic peptides corresponding to unique amino acid sequences: ClpP3, KVEGTKKDNTNLPERSMTQ; ClpP4, FEELDTTNMLLRQRI; and ClpP5, DIDI-QANEMLHHKANLNGYL. All Clp fusion proteins and conjugated synthetic peptides were injected into rabbits intramuscularly and subcutaneously (AgriSera, Hällnäs, Sweden).

Plant material, stress treatments and Chl fluorescence measurements

Arabidopsis thaliana plants (ecotype: Columbia) were grown in soil under controlled conditions: day/night temperatures 23/18°C, 8 h photoperiod, 62% humidity and 150 $\mu\text{mol m}^{-2}\text{s}^{-1}$ irradiance. Six-week-old plants used for all experiments were sampled 2 h into the photoperiod. For each stress, at least three replicate leaves were taken for each time point.

To test the effects of short-term severe stresses on the expression and synthesis of Clp proteins, detached *Arabidopsis* leaves were treated to different stress regimes. For each stress, all other growth conditions were kept constant unless stated. Desiccation stress was done by dehydrating leaves on Whatman 3 mm paper (Whatman Inc., NJ, USA) for 1 or 2 h at room temperature at an irradiance of 10 $\mu\text{mol m}^{-2}\text{s}^{-1}$. For salt and oxidative stress, leaves were floated on 200 or 400 mM NaCl, or 2% H_2O_2 solutions for 2 h, respectively. Wounding was done by rolling a metal brush across the detached leaves once (light) or several times (heavy) and then floated on water for 2 h. Cold and heat shock treatments were done by floating leaves on prechilled or preheated water inside growth cabinets at 5°C for 4 h and 37°C for 2 h, respectively. Extreme high light stress was applied to detached leaves for 4 h at 1500 $\mu\text{mol m}^{-2}\text{s}^{-1}$ as described by Heddad and Adamska (2000). Control leaves were also detached and floated on water under standard growth conditions for 2 or 4 h to test for changes in Clp proteins resulting from leaf excision.

For the longer, continuous high light treatment, whole plants were transferred from the standard irradiance of 150 $\mu\text{mol m}^{-2}\text{s}^{-1}$ to 850 $\mu\text{mol m}^{-2}\text{s}^{-1}$ for 1 d. Leaves were collected just prior to the shift (time 0 control), and then at 4, 8, 12 and 24 h of high light. Leaves from plants kept for 24 h at the standard irradiance of 150 $\mu\text{mol m}^{-2}\text{s}^{-1}$ were taken at the same time points as controls. For the prolonged high light, plants were shifted for 7 d to 850 $\mu\text{mol m}^{-2}\text{s}^{-1}$ but with the same photoperiod (8 h day/16 h night).

For cold acclimation, plants were transferred to day/night temperatures of 5/5°C, with the photoperiod and irradiance kept constant. As controls, several plants were kept at 23/18°C for the duration of the cold treatment.

Leaves developed at 23°C were taken 0 (control), 3, 7, 15 and 21 d after the cold shift; while fully expanded leaves from the new rosette developed at 5°C were also taken as the cold acclimated samples. Photochemical efficiency of PSII in leaves was measured for the high light and cold experiments as the F_v/F_M ratio as previously described (Ottander and Öquist 1991).

Leaf cellular protein extraction and chloroplast subfractionation

Total cell protein samples were isolated from leaves by grinding in liquid N_2 and transferring the frozen powder to 400 μl of solubilization buffer (100 mM sucrose, 50 mM Tris/Cl [pH 8.0], 1 mM EDTA, 1 mM Pefabloc, 20 mM DTT, 2% lithium dodecyl sulphate). After mixing, samples were heated at 75°C for 5 min and then centrifuged at approximately 14 000 g for 15 min at 4°C. Insoluble pellets were discarded, while supernatants containing solubilized proteins were kept for immunoblot analysis. Chl contents were determined spectrophotometrically (Porra et al. 1989). For the tissue specificity analysis, solubilized protein extracts were first precipitated in 80% acetone to remove Chl and then resuspended in solubilization buffer. Protein concentrations were determined by the Bradford method according to the manufacturer's protocol (Pierce). Isolation and subfractionation of intact chloroplasts from leaves were achieved on Percoll gradients as previously described (Clarke and Critchley 1992, Kunst 1998).

Polyacrylamide gel electrophoresis and immunoblot analysis

Denatured protein samples containing equal Chl (1 μg) were separated on precast 3–8% gradient Tris-Acetate (ClpC/D) or 12% linear Bis-Tris (ClpP) NuPAGE gels (Novex, San Diego, CA, USA). After separation, proteins were transferred electrophoretically to a polyvinylidene difluoride filter (Immobilon, Millipore, Bedford, MA, USA). *Arabidopsis* Clp proteins were detected using specific polyclonal antibodies as described above, except for ClpC, which is described earlier (Porankiewicz and Clarke 1997). Primary antibodies were detected with the horseradish peroxidase-linked, anti-rabbit secondary antibody from donkey (Amersham Pharmacia) and visualized by enhanced chemiluminescence (Amersham Pharmacia) on X-ray film.

RNA preparation and analysis

For analysis of transcript levels, total RNA was isolated using the TrizolTM reagent (Life Technologies, CA, USA) and treated with RNase-free DNase (Promega). RT-PCR was performed with the SuperScriptTM One-step RT-PCR kit Life Technologies, USA) using 4 ng of RNA for *clpP1*, 20 ng for *clpP3–6* and *clpC1*, and 100 ng for *clpC2* and *clpD*. These RNA concentrations were found in preliminary experiments to be within the linear

response range of the PCR amplification for each set of gene-specific primers. For all RT-PCR reactions, an extra control reaction was performed without reverse transcriptase but with *Taq* polymerase to detect possible DNA contamination. RT-PCR products were separated on 1.0–1.2% agarose gels and viewed with a ChemImager™ 4000 Low Light Imaging System (Alpha Innotech Corp., San Leandro, CA, USA). The identities of all RT-PCR products were confirmed by DNA sequencing.

Results

Identification of ClpC and ClpP homologues in *Arabidopsis*

The major Hsp100 protein in plant chloroplasts is ClpC, with two distinct, but closely related isomers in tomato, both predicted as being located in chloroplasts (Gottesman et al. 1990). A search of the *Arabidopsis* EST database revealed a cDNA coding for a ClpC-like protein, which we later obtained and completely sequenced. The cDNA contained a 2784-bp ORF encoding a 928-amino acid polypeptide. Prediction analysis of the N-terminus revealed a 92-amino acid chloroplast transit peptide. The complete protein has the two distinct ATP-binding domains characteristic of large Hsp100 proteins, as well as the spacer region of intermediate length and the two conserved N-terminal domains representative of the ClpC class (Schrimmer et al. 1996). The putative mature *Arabidopsis* protein has high sequence identity to ClpC in other plants (90–94%) and cyanobacteria (approximately 80%), whereas the match to ClpC in Gram positive bacteria (e.g. *B. subtilis*, 60%) and ClpA in Gram negative bacteria (e.g. *E. coli*, 41%) is much lower. *Arabidopsis* ClpC is also dissimilar to the other chloroplast-localized Hsp100 protein (ClpD, Kiyosue et al. 1993), being only 50% identical. Recently, a second ClpC isomer was identified in *Arabidopsis* (87% identity; Nakabayashi et al. 1999), and which has since been distinguished from the protein in this study (hereafter referred to as ClpC1) as ClpC2 (Adam et al. 2001).

A search of the *Arabidopsis* EST and genomic databases was also done for homologues of the ClpP proteolytic subunit. Genes coding for six putative ClpP proteins were subsequently identified, with the isomers recently designated as ClpP1–6 (Adam et al. 2001). Four extra genes coding for ClpP-like proteins were also identified in the *Arabidopsis* genome, but all four predicted polypeptides lacked the highly conserved catalytic triad characteristic of ClpP proteins (i.e., Ser-His-Asp). Because of this distinction, these four additional polypeptides have been designated as ClpR1–4 (Clarke 1999, Adam et al. 2001). Six other Clp-like sequences were also identified but all have features too divergent to classify as either ClpP/R or Hsp100. A detailed analysis of these sequences has recently been reported (Peltier et al. 2001).

At the time of our study, only partially sequenced cDNA clones were available for ClpP3–6. We obtained these cDNA clones and completed their DNA sequenc-

ing. The residues, Ser (194), His (219) and Asp (269), forming the proteolytic active site of the ClpP protease, were found in all six *Arabidopsis* ClpP proteins. Except for the plastid-encoded ClpP1, the five nuclear-encoded ClpP proteins all have N-terminal extensions that are strongly reminiscent of organellar transit peptides. According to predictions, ClpP3–6 with high probability possess transit peptides targeted to plastids. In contrast, the transit peptide for ClpP2 is predicted as mitochondrial, a localization we have since confirmed experimentally (Halperin et al. 2001).

Antibodies specific to plastid-localized Clp proteins

To further study the various Clp proteins in *Arabidopsis* predicted to be localized in plastids, we prepared a suite of specific polyclonal antibodies. All sera produced were tested against cell extracts from *Arabidopsis* leaves to determine specificity. An antibody was already available for ClpC, made to the N-terminal domain of the cyanobacterial ClpC protein from *Synechococcus* (Porankiewicz and Clarke 1997). In leaf extracts, a single 98 kDa protein was detected using the ClpC antibody (Fig. 1A). However, because of the highly conserved nature of this N-terminal domain among ClpC homologues, and the near identity between ClpC1 and –2 in this region, the antibody detects both *Arabidopsis* isomers without distinction. For the other plastidic Hsp100 protein, ClpD, an antibody was made to the C-terminal domain downstream of the second highly conserved ATP-binding domain. The resultant antibody detected a protein matching the predicted mature size of ClpD (approximately 102 kDa) in *Arabidopsis* leaf cell extracts. The antibody also weakly detected a slightly smaller protein corresponding to the size of ClpC (Fig. 1A) likely due to slight cross-reaction to the more abundant ClpC protein.

Besides the Hsp100 proteins, polyclonal antibodies were made for each of the *Arabidopsis* ClpP isomers. Each of the antibodies detected a single ClpP protein of the expected molecular mass (ClpP1, 20.5 kDa; ClpP3, 28.5 kDa; ClpP4, 27 kDa; ClpP5, 22.5 kDa; ClpP6, 21.5 kDa) without observable cross-reaction to any of the other ClpP isomers (Fig. 1B). In the case of ClpP3 and -P5, slight cross-reaction to proteins larger than 35 kDa was observed, although these did not interfere with the detection of the ClpP isomer in this study.

Localization of Clp proteins in chloroplast stroma

With the identification of new ClpP isomers in *Arabidopsis*, we investigated whether they were localized in chloroplasts as was predicted by their transit peptide sequences. Intact chloroplasts were purified from young *Arabidopsis* leaves and fractionated into stromal and thylakoid membrane/luminal proteins. Using our specific antibodies, all investigated Clp proteins were detected in intact chloroplasts in amounts similar to whole leaf extracts (Fig. 2), indicating chloroplast localization for each Clp protein. Within chloroplasts, all Clp proteins

were localized in the stromal fraction, with no detectable signal in the thylakoid membrane fraction. Polyclonal antibodies raised against Lhcb2, a known thylakoid membrane protein, were used as a control to confirm the integrity of the thylakoid subfraction. These results demonstrate that the three newly identified nuclear-encoded ClpP isomers (ClpP3–5) are transported into chloroplasts and localized in the stroma along with ClpC, –D, –P1 and –P6.

Tissue-specific expression and synthesis of Clp proteins

To study the regulatory characteristics of the chloroplast Clp proteins mentioned above, tissue from roots, stems and leaves were collected to test for potential differential gene expression and protein synthesis. Levels of *Arabidopsis* clp-specific mRNA and protein were analysed by RT-PCR and immunoblotting, respectively. Similar transcript levels were observed for the *hsp100* genes (*clpC1*, –

C2 and –*D*) in the three tissue types, whereas the corresponding protein contents were relatively low in roots but steadily increased from stems to leaves (Fig. 3). This discrepancy between mRNA and protein levels suggests either post-transcriptional regulation of *hsp100* transcripts reducing protein levels in root and stem cell plastids, or increased stability of chloroplast Hsp100 proteins in leaf cells. In contrast, transcript and protein levels for most *Arabidopsis* clpP genes were both low in roots and increased steadily from stems to leaves (Fig. 3), suggesting little if any post-transcriptional regulation. The one exception was *clpP5*, for which transcript levels changed less dramatically in the different tissues than protein content.

Levels of Clp proteins under stress conditions

Clp proteins in different bacteria are involved in many stress responses, such as heat, high salt, low temperature, and UV light, and often strongly induced after brief exposures (Porankiewicz et al. 1999). Given the many Clp

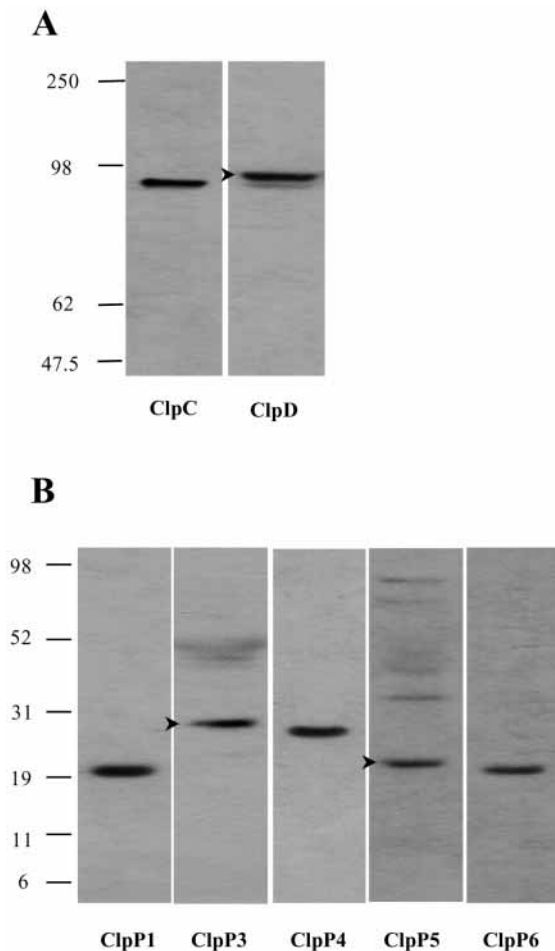


Fig. 1. Specific polyclonal antibodies against *Arabidopsis* Clp proteins. Cell extracts from *Arabidopsis* leaves were separated by denaturing PAGE optimized for large Hsp100 protein (A) or smaller ClpP isomers (B). Immunoblot analysis was then performed with each serum on replicate samples to test the specificity of each individual antibody. Specificity of the antibodies is shown at the bottom of each lane, with molecular mass standards in kDa shown on the left.

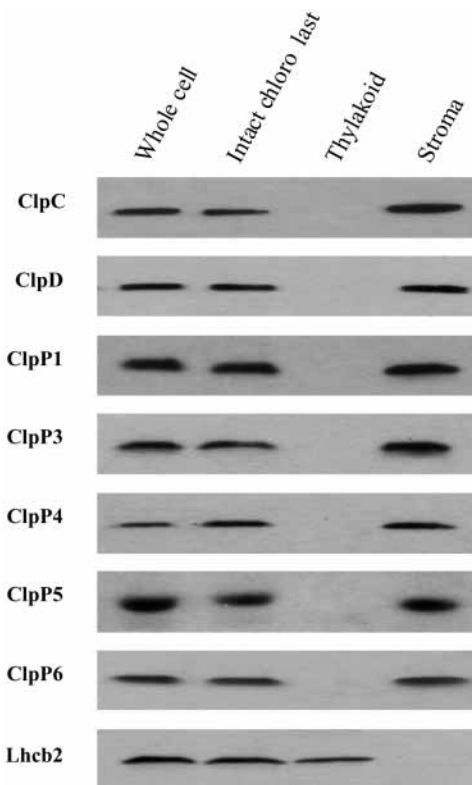


Fig. 2. Chloroplast localization of Clp proteins in *Arabidopsis*. The intracellular location of the ClpP3–5 proteins was compared to that of the previously studied ClpC, –D, –P1 and –P6 proteins. Proteins from whole cell extracts (lane 1), intact chloroplasts (lane 2), thylakoid membranes (lane 3) and soluble stroma (lane 4) are isolated and fractionated from *Arabidopsis* leaves. Samples were separated by denaturing PAGE on the basis of equal Chl content (1 µg, lanes 1–3) or equivalent protein content (5 µg, lane 4), and then analysed by immunoblotting using antibodies specific for each Clp protein as indicated on the left. Also included was the known thylakoid membrane, Lhcb2, as a control for the chloroplast subfractionation.

proteins in *Arabidopsis* chloroplasts, we examined if any could be involved in similar stress responses. For this we studied the potential stress regulation of chloroplast Clp proteins at different levels; possible rapid protein induction upon moderate or severe stress treatments, and induction during longer acclimation to high light and cold.

Several moderate and severe stresses including dehydration, high salt concentration, heat and cold shock, oxidation, wounding and high light intensity were briefly applied to detached *Arabidopsis* leaves. In addition to the time zero control, controls detached for 2 and 4 h were also included to exclude possible increases in Clp protein synthesis resulting solely from leaf excision. Under certain stress conditions, decreased Clp protein content was observed, especially for ClpP3 after wounding and high light treatments (Fig. 4). However, no significant and reproducible induction of any chloroplast Clp protein occurred during the various stresses, suggesting most if not all, are mainly constitutive proteins in *Arabidopsis*.

High light stress response

Because of the lack of any obvious rapid induction of chloroplast Clp proteins in response to brief but severe

stresses, we investigated more prolonged moderate stresses. High light intensity was chosen as the first model stress since we had previously shown strong induction of Clp proteins in cyanobacteria during extended photoinhibitory conditions (Clarke et al. 1998). Initially we tested for possible diurnal expression of *clp* genes in *Arabidopsis* to reveal any changes over the 24 h time period chosen for the first high light treatment. Diurnal expression was studied for ClpP1–6, ClpC1–2 and ClpD, but no significant changes in both transcript and protein contents were observed throughout the 24-h period (data not shown), indicating no significant diurnal rhythms for these *clp* genes. For the first high light experiments, *Arabidopsis* plants grown at $150 \mu\text{mol m}^{-2} \text{s}^{-1}$ were shifted to $850 \mu\text{mol m}^{-2} \text{s}^{-1}$ for 24 h, with all other growth conditions kept constant. Several *Arabidopsis* plants were also kept at $150 \mu\text{mol m}^{-2} \text{s}^{-1}$ for 24 h as a control, from which later analysis showed no significant change in Clp protein levels throughout the time course. The degree of high light stress in leaves was monitored by the Chl fluorescence parameter F_V/F_M for photosynthetic efficiency. Prior to the high light shift, control leaves had an F_V/F_M of 0.843 (± 0.003 SE, $n = 7$), which then fell steadily to 0.711 (± 0.012 SE, $n = 12$) after 8 h of high light, and remained at this low level for

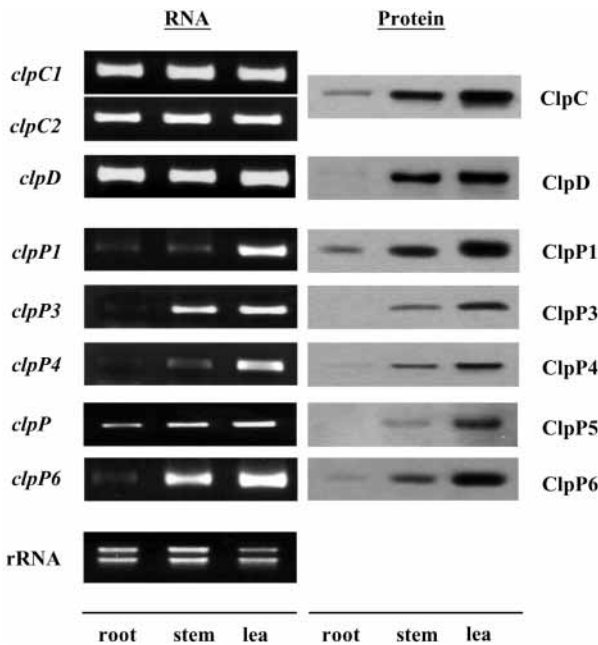


Fig. 3. Tissue specificity of mRNA and protein levels of plastid-localized Clp proteins in *Arabidopsis*. Comparative transcript levels for each *clp* gene in *Arabidopsis* roots, stems and leaves were determined by RT-PCR from equal amounts of total RNA. RT-PCR products were separated on agarose gels and visualized by ethidium bromide staining. Equivalent amounts of total rRNA for each set of RT-PCR reactions were confirmed by staining of 25S and 18S rRNA. Comparative Clp protein levels in root, stem and leaf tissues were determined by immunoblotting using the specific polyclonal antibodies. For each tissue, whole cell proteins were extracted and separated by denaturing PAGE on the basis of equal protein (10 μg). The figure shows results representative of three replicates.

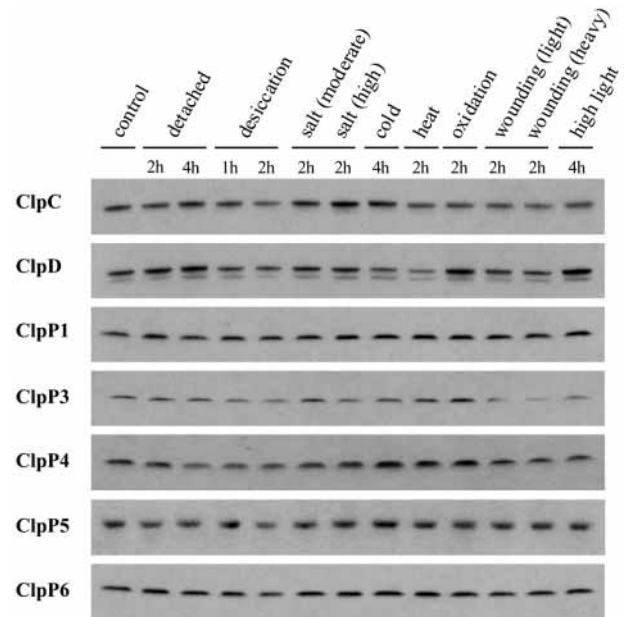


Fig. 4. Levels of chloroplast Clp proteins in *Arabidopsis* exposed to short-term severe stresses. Detached leaves from six-week-old plants were treated with different severe stresses for brief periods: 1. attached control, 2. control detached (2 h), 3. control detached (4 h), 4. desiccation (1 h), 5. desiccation (2 h), 6. 200 mM NaCl (2 h), 7. 400 mM NaCl (2 h), 8. 5°C (4 h), 9. 42°C (2 h), 10. 2% H_2O_2 (2 h), 11. light wounding (2 h), 12. heavy wounding (2 h), 13. high light (4 h). Cellular proteins were extracted after each treatment and separated by denaturing PAGE on the basis of equal Chl (1 μg). Individual Clp proteins were detected by immunoblotting with specific antibodies. The figure shows results representative of three independent replicates.

the remaining treatment. ClpC, ClpP1, -3, -5 and -6 content did not significantly change during the 24 h high light. However, levels of ClpD and -P4 proteins both declined (approximately 50%) after the high light shift, but then completely recovered within the 24-h period (data not shown).

The second high light treatment was a shift in day irradiance from 150 to 850 $\mu\text{mol m}^{-2}\text{s}^{-1}$ for 7 d, with photoperiod and other conditions unchanged. F_v/F_M values showed that plants had recovered from the early photoinhibitory stress by 3 d, which then remained unchanged by 7 d. Chl and fresh wt also remained constant throughout the high light treatment. The amount of ClpC, -P3, -P4 and -P6 did not significantly change in leaves during the 7 d high light regime (Fig. 5). In contrast, leaf ClpP1 content increased several fold from 3 to 7 d, while ClpP5 levels rose steadily in both the 3 and 7 d samples. ClpD protein content was also strongly induced after 3 d at 850 $\mu\text{mol m}^{-2}\text{s}^{-1}$, but this returned to control levels by 7 d (Fig. 5).

Cold acclimation

Because of the induction of certain Clp proteins during the prolonged high light treatment, we analysed their levels during acclimation of plants to another moderate stress condition. Cold acclimation was selected, again because of earlier observed Clp protein induction during cold acclimation in cyanobacteria (Porankiewicz and

Clarke 1997, Porankiewicz et al. 1998). Six-week-old *Arabidopsis* plants were shifted from 23/18°C to 5/5°C day/nights with unchanged photoperiod, irradiance and humidity. Leaves were collected just prior to the shift (0 h control) and then after 3, 7, 15, 21 and 42 d at 5°C. Leaves from another set of plants kept at 23/18°C were also taken at these times as extra controls, except at 42 d when leaves from the second rosette dominated and shaded the mature, first rosette underneath. The degree of cold stress in plants shifted to 5°C was monitored throughout by F_v/F_M . In leaves developed at 23°C prior to the cold shift (warm-developed), F_v/F_M dropped from control values of 0.841 (± 0.004 SE, $n = 6$) to 0.792 (± 0.007 SE, $n = 9$) after 1 d at 5°C, and remained at this level until day 7, after which it slowly recovered to 0.820 (± 0.005 SE, $n = 12$) after 21 d. At 42 d, F_v/F_M of new leaves developed at 5°C from the second rosette (0.848 ± 0.004 SE, $n = 9$) was the same as for warm-developed control leaves, indicating the shifted plants had acclimated to the low temperature.

Potential changes in *clp* gene expression and protein content were analysed in *Arabidopsis* leaves during the cold acclimation (Fig. 6). Of the *hsp100* genes, there were neither significant changes in transcript levels for *clpC1* or -C2, nor any change in total ClpC protein content. In contrast, levels of *clpD* mRNA and protein in warm-developed leaves increased markedly from 3 to 7 d at 5°C, and remained high at 15 and 21 d. The *clpD* mRNA and protein also remained at this elevated level in the cold-developed leaves at 42 d, suggesting a role for ClpD during cold acclimation. For the *clpP* genes, there were no significant changes in mRNA levels during the cold treatment except for *clpP3*. Transcripts of *clpP3* increased 2- to 3-fold in warm-developed leaves in the first 3 d and stayed at this level until 21 d, although in cold-developed leaves (42 d) it was slightly lower. This increase in transcript level was followed by an increase in ClpP3 protein after 7 d, which rose further by 21 d and in cold-developed leaves at 42 d. For the other ClpP isomers, the amounts of ClpP1, -3, -5 and -6 protein significantly increased after 7 or 15 d at 5°C, despite no obvious changes in mRNA level, and all remained at this elevated level after 21 d and in cold-developed leaves at 42 d. Only ClpP4 protein content remained unaffected by the cold treatment, which mirrored its mRNA profile (Fig. 6).

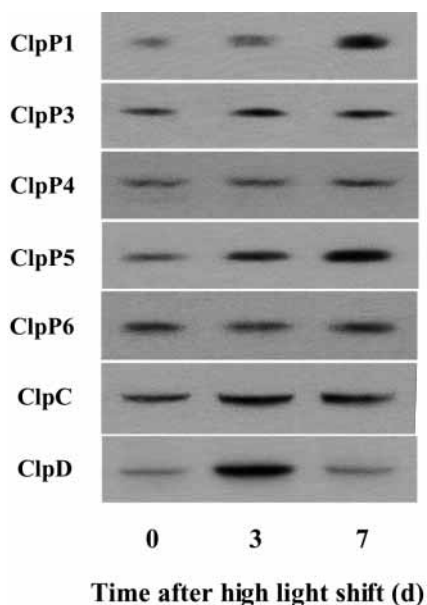


Fig. 5. Level of chloroplast Clp proteins in *Arabidopsis* leaves exposed to prolonged high light. Six-week-old *Arabidopsis* plants were shifted from 150 to 850 $\mu\text{mol m}^{-2}\text{s}^{-1}$, with the same photoperiod, temperature and humidity. Leaves from the same rosette were taken just prior to (0 h control) and 3 and 7 d after the change in irradiance. Individual Clp proteins were detected by immunoblotting with specific antibodies and compared on the basis of equal Chl (1 μg) content. The figure shows results representative of three independent replicates.

Discussion

In this study we describe several new *clp* genes in *Arabidopsis*, one coding for a ClpC homologue (ClpC1) and three for distinct ClpP isomers (ClpP3–5). The addition of these genes now increases the total number of confirmed *clp* genes in *Arabidopsis* to 19, by far the greatest number as yet found in any organism. Moreover, six other Clp-like sequences have also recently been identified in *Arabidopsis* (Peltier et al. 2001) from the recently completed genome sequence, thereby possibly further increasing the complexity of plant Clp proteins.

Of the recognized polypeptides, nine are members of the Hsp100/Clp family of molecular chaperones (4 ClpB, 2 ClpC, 1 ClpD and 2 ClpX), six are homologues of the ClpP proteolytic subunit, and four are ClpP-like proteins now termed ClpR. To clarify this situation, we have recently proposed a standardized nomenclature for most of these Clp proteins (Adam et al. 2001), which we have conformed to in this study.

Most of the Clp proteins in *Arabidopsis* are localized in chloroplasts. We were able to demonstrate the stromal localization of the new ClpP isomers, ClpP3–5, and confirm similar localizations for ClpC, -D, -P1 and -P6 (Shanklin et al. 1995, Nakabayashi et al. 1999). Of the

remaining Clp proteins, ClpR1–3 also appear to be localized in chloroplasts (Nakabayashi et al. 1999, Peltier et al. 2001), while ClpX1, -X2 and -P2 are found in mitochondria (Halperin et al. 2001). The intracellular location of ClpR4 remains unknown. All the stromal Clp proteins in this study almost certainly function in ATP-dependent Clp proteases although direct evidence for such remains absent. It is equally plausible that the chloroplastic Hsp100 proteins also perform chaperone roles independent of ClpP proteolysis, as do the Hsp100 homologues in other organisms (Wickner et al. 1994, Levchenko et al. 1995), with an involvement in chloroplast protein import being one such possible function (Nielsen et al. 1997). With the addition of ClpP3–5 proteolytic subunits for stromal Clp proteases. Why so many ClpP isomers occur in chloroplasts and whether they form homogeneous or heterogeneous ClpP complexes remains unclear. Since it is the Hsp100 chaperone subunit that confers substrate specificity to the Clp protease and regulates its activity, there seems no obvious explanation why plants possess more than one ClpP in chloroplasts. Based on structural comparisons of the three ClpP isomers in cyanobacteria (Porankiewicz et al. 1999), we proposed they might have different affinities for different Hsp100 partners. If true for chloroplast ClpP proteins, then more than one isomer is likely to associate with each Hsp100 protein since only three probable Clp regulatory subunits exist in chloroplasts (ClpC1, -C2 and -D). An alternative possibility is that some chloroplast ClpP proteins form proteolytic complexes with other chaperones like Hsp60, thereby increasing the number of potential regulatory partners and broadening the range of protein substrates. The bacterial Hsp60 (GroEL) is known to activate the proteolytic activity of ClpP in *E. coli* in the absence of Hsp100 proteins (Kandrór et al. 1994), and Hsp60 is one of the major molecular chaperones in the chloroplast stroma. An equally important issue to resolve is the role of ClpR polypeptides in chloroplasts and whether they interact with ClpP isomers or Hsp100 proteins.

Before this study it was unknown whether the numerous Clp proteins in chloroplasts exhibited differential gene expression/protein synthesis under different physiological conditions. Given that such variations in the synthesis of each Clp protein type would greatly affect the composition and substrate specificity of potential chloroplast Clp proteases, it was crucial to perform a more comprehensive analysis than had been previously attempted. For this we combined the powerful techniques of RT-PCR and immunoblotting to compare mRNA and protein levels for each Clp protein in different tissues and growth regimes. In terms of tissue specificity, all the studied plastid Clp proteins showed similar protein profiles, being most abundant in leaves and relatively low in roots. This suggests that all plastid localized Clp proteins are most abundant in chloroplasts, with levels highest in tissues with the greatest chloroplast density, as seen when comparing stems and leaves. In the

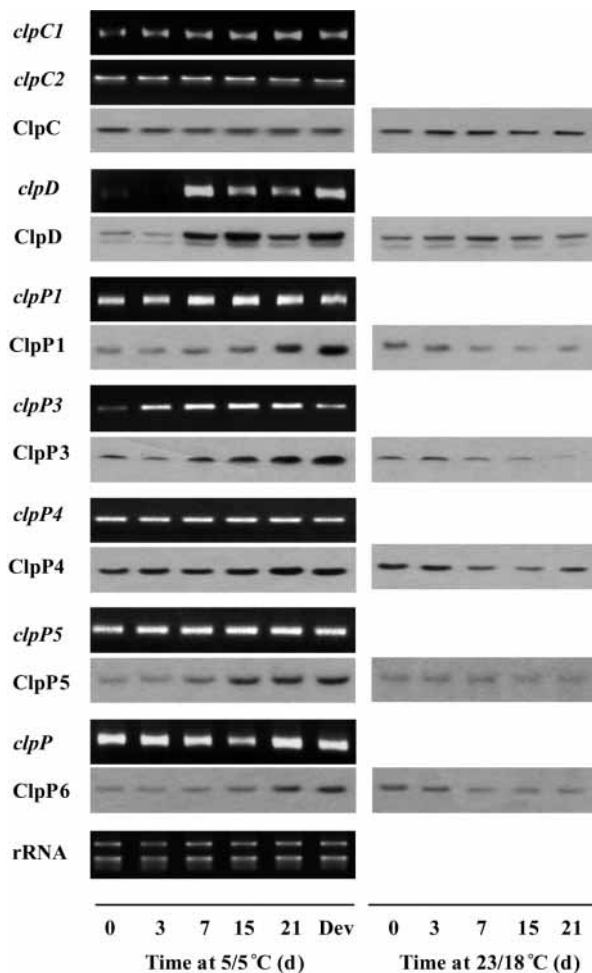


Fig. 6. *Arabidopsis* chloroplast Clp proteins during cold acclimation. Plants growing at 23/18°C were shifted to 5/5°C with the same photoperiod and irradiance. Plants were chilled at 5°C until cold-acclimated leaves from the second rosette were fully expanded. Leaves from the same rosette developed at 23°C were taken prior to (0 h control) and 3, 7, 15 and 21 d after the cold shift. Mature leaves from the new rosette developed at 5°C were also harvested as the acclimated samples (Dev). Levels of mRNA and protein for each Clp protein were determined by RT-PCR and immunoblotting on the basis of equal total RNA (10 µg) or Chl (1 µg) content. Leaves from plants kept under standard conditions for the first 21 d of cold were also examined for possible changes in protein content during senescence. The figure shows results representative of three independent replicates.

case of *clpP1*, the higher plastome copy number in chloroplasts may also contribute to greater amounts of ClpP1 protein in leaves relative to roots. Despite their low concentrations, however, all plastid Clp proteins were present in roots, thereby excluding a role for Clp proteases exclusive to photosynthetic tissues. This supports earlier proposals that Clp proteases in plants may at one level perform housekeeping duties within plastids and be involved in the turnover of proteins with functions unrelated to photosynthesis (Clarke 1999).

The tissue specificity study also revealed distinct regulatory differences between the plastid Hsp100 and most ClpP proteins. For the Hsp100 proteins, the transcript levels for *clpC1*, *-C2* and *-D* were the same in all three tissues, despite the considerably higher protein levels in stems and leaves. This suggests significant post-transcriptional and/or translational regulation exists in *Arabidopsis* that restricts the synthesis of Hsp100 proteins in root cells. In contrast, the mRNA profiles for the *clpP* genes closely matched the protein profiles, indicating that ClpP protein contents in plastids is regulated primarily at the transcriptional level in *Arabidopsis*. The one exception to this was *clpP5*, which exhibited transcript levels similar to those for the *hsp100* genes, also suggesting a degree of post-transcriptional regulation. At this stage the reason for such differential regulation of *clp* gene expression in *Arabidopsis* remains unclear.

Besides being abundant in leaves, the constitutive level of chloroplast Clp proteins did not dramatically change during transient severe and moderate stresses. Although not exhaustive, many different severe stress conditions were examined. Under all these conditions, however, no significant induction occurred for any of the chloroplast Clp proteins. This contrasts with studies in various eubacteria, in which ClpC or *-P* proteins are rapidly induced by the same stress regimes used in this study (Porankiewicz et al. 1999). In plants, a similar absence of stress induction as shown in this study was also observed for ClpP1 and ClpC during short-term heat shock, chilling and dehydration (Shanklin et al. 1995, Ostersetzer and Adam 1996). In the case of ClpC, this lack of stress induction also occurred for the closely related homologue in cyanobacteria (Clarke and Eriksson 1996). For ClpD, previous studies have reported rapid induction of *clpD* gene expression in *Arabidopsis* leaves during severe desiccation and high salt stress, and during natural senescence and dark-induced etiolation (Kiyosue et al. 1993, Nakashima et al. 1997), although in our study such stresses failed to significantly increase amounts of ClpD protein. This apparent discrepancy between mRNA and protein levels has recently been observed for ClpD during senescence, in which levels of ClpD protein actually decreased in *Arabidopsis* leaves concomitant to increased *clpD* transcripts (Weaver et al. 1999), suggesting that considerable post-transcriptional regulation exists for this *clp* gene like that observed in *Arabidopsis* roots. Overall, the lack of major increases in levels of chloroplast Clp proteins suggests none of the tested proteins are rapidly induced stress proteins in leaves. It must

be noted, however, that in other tissues like roots with low constitutive levels of Clp protein, the possibility remains of stress induction for one or more isomers.

In contrast to the short-term stresses, prolonged high light and cold treatment did result in significantly increased levels of certain chloroplast Clp proteins, including ClpD and several ClpP isomers. In the case of ClpP, both ClpP1 and *-P5* were induced during the latter stages of both high light and cold shifts, suggesting the two proteins may be involved in acclimation to each new growth condition. It should be noted that for the high light shift the acclimation was to a more desirable light intensity whereas the cold shift was to a less favourable temperature. ClpD would also appear involved in these acclimation processes, being the earliest induced Clp protein under both conditions and at levels highest in leaves recovering from the initial stress period. As leaves acclimatise to the new condition, ClpD content declines as after 7 d at high light and 21 d at 5°C. In the case of cold, however, ClpD content remained high in leaves developed at 5°C, suggesting a more prolonged involvement by this chloroplast protein in cold hardiness. This induction of ClpD during cold acclimation is the first report where ClpD levels rise concomitantly with *clpD* mRNA in response to an altered growth condition or developmental transition. Given that the induced ClpD and ClpP proteins likely function in Clp proteolytic complexes, their accumulation suggests increased chloroplast protein turnover is associated with both high light and cold acclimation.

The nucleotide sequences reported in this paper have been submitted to the GenBank/EBI Data Bank with accession numbers AF016621, AF022909 and AJ012278.

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References

- Adam Z, Adamska I, Nakabayashi K, Ostersetzer O, Haussuhl K, Manuell A, Zheng B, Vallon O, Rodermel SR, Shinozaki K, Clarke AK (2001) Chloroplast and mitochondrial proteases in *Arabidopsis thaliana*: a proposed nomenclature. *Plant Physiol* 125: 1912–1918.
- Clarke AK (1999) ATP-dependent Clp proteases in photosynthetic organisms – A cut above the rest! *Ann Bot* 83: 593–599
- Clarke AK, Critchley C (1992) The identification of a heat-shock protein complex in chloroplasts of barley leaves. *Plant Physiol* 100: 2081–2089
- Clarke AK, Eriksson M-J (1996) The cyanobacterium *Synechococcus* sp. PCC 7942 possesses a close homologue to the chloroplast ClpC protein of higher plants. *Plant Mol Biol* 31: 721–730
- Clarke AK, Schelin J, Porankiewicz J (1998) Inactivation of the *clpI* gene for the proteolytic subunit of the ATP-dependent Clp

- protease in the cyanobacterium *Synechococcus* limits growth and light acclimation. *Plant Mol Biol* 37: 791–801
- Desimone M, Weiss-Wichert W, Wagner E, Altenfeld U, Johanningmeier U (1997) Immunohistochemical studies on the Clp-protease in chloroplasts: evidence for the formation of a ClpC/P complex. *Bot Acta* 110: 234–239
- Emanuelsson O, Nielsen H, von Heijne G (1999) ChloroP, a neural network-based method for predicting chloroplast transit peptides and their cleavage sites. *Protein Sci* 8: 978–984
- Emanuelsson O, Nielsen H, Brunak S, von Heijne G (2000) Predicting subcellular localization of proteins based on their N-terminal amino acid sequence. *J Mol Biol* 300:1005–1016
- Gottesman S, Squires C, Pichersky E, Carrington M, Hobbs M, Mattick JS, Dalrymple B, Kuramitsu H, Shiroza T, Foster T, Clark WP, Ross B, Squires CL, Maurizi, MR (1990) Conservation of the regulatory subunit for the Clp ATP-dependent protease in prokaryotes and eukaryotes. *Proc Natl Acad Sci USA* 87: 3513–3517
- Grimaud R, Kessel M, Beuron F, Stevens AC (1998) Enzymatic and structural similarities between the *Escherichia coli* ATP-dependent proteases, ClpXP and ClpAP. *J Biol Chem* 273: 12476–12481
- Halperin T, Zheng B, Itzhaki H, Clarke AK, Adam Z (2001) Plant mitochondria contain proteolytic and regulatory subunits of the ATP-dependent Clp protease. *Plant Mol Biol* 45: 461–468
- Haußühl K, Andersson B, Adamska I (2001) A, chloroplast DegP and 2 protease performs the primary cleavage of the photodamaged, D. 1 protein in plant photosystem II. *EMBO J* 20: 713–722
- Heddad M, Adamska I (2000) Light stress-regulated two-helix proteins in *Arabidopsis thaliana* related to the chlorophyll *alb*-binding gene family. *Proc Natl Acad Sci USA* 97: 3741–3746
- Itzhaki H, Naveh L, Lindahl M, Cook M, Adam Z (1998) Identification and characterization of DegP, a serine protease associated with the luminal side of the thylakoid membrane. *J Biol Chem* 273: 7094–7098
- Kandror O, Busconi L, Sherman M, Goldberg AL (1994) Rapid degradation of an abnormal protein in *Escherichia coli* involves the chaperones GroEL and GroES. *J Biol Chem* 269: 23575–23582
- Kiyosue T, Yamaguchi-Shinozaki K, Shinozaki K (1993) Characterization of cDNA for a dehydration-inducible gene that encodes a CLP A, B-like protein in *Arabidopsis thaliana* L. *Biochem Biophys Res Commun* 196: 1214–1220
- Kunst L (1998) Preparation of physiologically active chloroplasts from *Arabidopsis*. In: Martinez-Zapater JM, Salinas J (eds) *Methods in Molecular Biology. Arabidopsis protocols*, Vol 82 Humana Press Inc, NJ, pp 43–48
- Levchenko I, Luo L, Baker, TA (1995) Disassembly of the Mu transposase tetramer by the ClpX chaperone. *Genes Dev* 9: 2399–2408
- Lindahl M, Tabak S, Cseke L, Pichersky E, Andersson B, Adam Z (1996) Identification, characterization, and molecular cloning of a homologue of the bacterial FtsH protease in chloroplasts of higher plants. *J Biol Chem* 271: 29329–29334
- Moore T, Keegstra, K (1993) Characterization of a cDNA clone encoding a chloroplast-targeted Clp homologue. *Plant Mol Biol* 21: 525–537
- Nakabayashi K, Ito M, Kiyosue T, Shinozaki K, Watanabe, A (1999) Identification of clp genes expressed in senescing *Arabidopsis* leaves. *Plant Cell Physiol* 40: 504–514
- Nakashima K, Kiyosue T, Yamaguchi-Shinozaki K, Shinozaki K (1997) A nuclear gene, *erd1*, encoding a chloroplast-targeted Clp protease regulatory subunit homolog is not only induced by water stress but also developmentally up-regulated during senescence in *Arabidopsis thaliana*. *Plant J* 12: 851–861
- Nielsen E, Akita M, Davila-Aponte J, Keegstra K (1997) Stable association of chloroplastic precursors with protein translocation complexes that contain proteins from both envelope membranes and a stromal Hsp100 molecular chaperone. *EMBO J* 16: 935–946
- Ostersetzer O, Adam Z (1996) Effects of light and temperature on expression of ClpC, the regulatory subunit of chloroplastic Clp protease, in pea seedlings. *Plant Mol Biol* 31: 673–676
- Ottander C, Öquist G (1991) Recovery of photosynthesis in winter-stressed Scots pine. *Plant Cell Physiol* 14: 345–349
- Parsell DA, Lindquist S (1993) The function of heat-shock proteins in stress tolerance: degradation and reactivation of damaged proteins. *Annu Rev Genet* 27: 437–496
- Peltier JB, Ytterberg J, Liberles DA, Roepstorff P, van Wijk KJ, (2001) Identification of a, 350 kDa ClpP and protease complex with, 10 different Clp isoforms in chloroplasts of *Arabidopsis thaliana*. *J Biol Chem* 276: 16318–16327
- Porankiewicz J, Clarke AK (1997) Induction of the heat shock protein ClpB affects cold acclimation in the cyanobacterium *Synechococcus* sp. Strain PCC 7942. *J Bacteriol* 179: 5111–5117
- Porankiewicz J, Schelin J, Clarke AK (1998) The ATP-dependent Clp protease is essential for acclimation to UV-B and low temperature in the cyanobacterium *Synechococcus*. *Mol Microbiol* 29: 275–283
- Porankiewicz J, Wang J, Clarke AK (1999) New insights into the ATP-dependent Clp protease: *Escherichia coli* and beyond. *Mol Microbiol* 32: 449–458
- Porra RJ, Thompson WA, Kriedemann PE (1989) Determination of accurate extinction coefficients and simultaneous equations for assaying chlorophylls a and b extracted with four different solvents. *Biochim Biophys Acta* 975: 384–394
- Riggs P (1990) Expression and purification of maltose-binding protein fusions. In: Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K (eds) *Current Protocols in Molecular Biology*. Greene Publishing Associates Inc, New York
- Schirmer EC, Lindquist S, Vierling E (1994) An *Arabidopsis* heat shock protein complements a thermotolerance defect in yeast. *Plant Cell* 6: 1899–1909
- Schirmer EC, Glover JR, Singer MA, Lindquist S (1996) HSP100/Clp proteins: a common mechanism explains diverse functions. *TIBS* 21: 289–295
- Shanklin J, Dewitt ND, Flanagan JM (1995) The stroma of higher plant plastids contain ClpP and ClpC, functional homologs of *Escherichia coli* ClpP and ClpA: an archetypal two-component ATP-dependent protease. *Plant Cell* 7: 1713–1722
- Shikanai T, Shimizu K, Ueda K, Nishimura Y, Kuroiwa T, Hashimoto T (2001) The chloroplast *clpP*, gene, encoding a proteolytic subunit of, ATP, -dependent protease and is indispensable for chloroplast development in tobacco. *Plant Cell Physiol* 42: 264–273
- Singh SK, Grimaud R, Hoskins JR, Wickner S, Maurizi MR (2000) Unfolding and internalization of proteins by the ATP-dependent proteases ClpXP and ClpAP. *Proc Natl Acad Sci USA* 97: 8898–8903
- Sokolenko A, Lerbs-Mache S, Altschmied L, Herrmann RG (1998) Clp protease complexes and their diversity in chloroplasts. *Planta* 207: 286–295
- Wang J, Hartling JA, Flanagan JM (1997) The structure of ClpP at 2.3 Å resolution suggests a model for ATP-dependent proteolysis. *Cell* 91: 447–456
- Weaver LM, Froehlich JE, Amasino RM (1999) Chloroplast-targeted ERD1 protein declines but its mRNA increases during senescence in *Arabidopsis*. *Plant Physiol* 119: 1209–1216
- Wickner S, Gottesman S, Skowyra D, Hoskins J, McKenney K, Maurizi, MR (1994) A molecular chaperone, ClpA, functions like DNAK and DNAJ. *Proc Natl Acad Sci USA* 91: 12218–12222