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The chloroplast protease subunit ClpP4 is a substrate of the E3 ligase AtCHIP and plays an important role in chloroplast function

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Summary

Animal CHIP proteins are chaperone-dependent E3 ubiquitin ligases that physically interact with Hsp70, Hsp90 and proteasome, promoting degradation of a selective group of non-native or damaged proteins in animal cells. The plant CHIP-like protein, AtCHIP, also plays important roles in protein turnover metabolism. AtCHIP interacts with a proteolytic subunit, ClpP4, of the chloroplast Clp protease *in vivo*, and ubiquitylates ClpP4 *in vitro*. The steady-state level of ClpP4 is reduced in *AtCHIP*-overexpressing plants under high-intensity light conditions, suggesting that AtCHIP targets ClpP4 for degradation and thereby regulates the Clp proteolytic activity in chloroplasts under certain stress conditions. Overexpression of *ClpP4* in Arabidopsis leads to chlorotic phenotypes in transgenic plants, and chloroplast structures in the chlorotic tissues of *ClpP4*-overexpressing plants are abnormal and largely devoid of thylakoid membranes, suggesting that ClpP4 plays a critical role in chloroplast structure and function. As AtCHIP is a cytosolic protein that has been shown to play an important role in regulating an essential chloroplast protease, this research provides new insights into the regulatory networks controlling protein turnover catabolism in chloroplasts.

Keywords: chaperone co-factor, E3 ligase, chloroplast protease, protein degradation, ubiquitylation.

Introduction

The mechanism that maintains cellular protein homeostasis is called protein quality control (Gottesman *et al.*, 1997). Molecular chaperones, ubiquitylation enzymes and the 26S proteasome are the major players in protein homeostasis (Wickner *et al.*, 1999). Over the years, we have learned a great deal about protein folding and turnover metabolisms, and have identified most, if not all, components of various chaperone molecules, the ubiquitylation system, and the protein degradation machinery, the 26S proteasome, in eukaryotic cells (Ciechanover *et al.*, 2000; Ferrell *et al.*, 2000; Smalle and Vierstra, 2004). The actions of several chaperone co-factors influence the direction of the pathway that a non-native protein would take: refolding or degradation. One of the chaperone co-factors in protein quality control is CHIP, which binds to the C-terminus of a mouse chaperone protein Hsc70. Binding of CHIP inhibits the ATPase activity of Hsp70 and its substrate binding, preventing other co-chaperones from binding to Hsp90, in turn inhibiting the protein folding activities of both Hsp70 and Hsp90 (Ballinger *et al.*, 1999; Connell *et al.*, 2001; Meacham *et al.*, 2001). Furthermore, CHIP physically links Hsp70 and Hsp90 to the 26S proteasome and promotes degradation of the substrate proteins of Hsp70/Hsp90 (Ballinger *et al.*, 1999; Connell *et al.*, 2001; Meacham *et al.*, 2001). CHIP proteins are U-box-containing E3 ubiquitin ligases that induce ubiquitylation of the substrates of Hsp70 and Hsp90 and stimulate their degradation through the 26S proteasome (Connell *et al.*, 2001; Demand *et al.*, 2001; Meacham *et al.*, 2001). Therefore, CHIP functions largely as a degradation factor in protein turnover metabolism and plays a key role in cellular protein homeostasis, whereby accumulation of misfolded polypeptides to toxic levels is prevented through refolding (via chaperones) or degradation (via proteasome; Cyr *et al.*, 2002; Höhfeld *et al.*, 2001; Murata *et al.*, 2001).

CHIP homologs have been found in most eukaryotes, suggesting that their function is evolutionarily conserved (Patterson, 2002). Recently, we characterized an Arabidopsis protein, AtCHIP, that is structurally similar to animal CHIPs with three tetratricopeptide repeats (TPRs) and one U-box domain (Yan *et al.*, 2003). The TPR is involved in protein–protein interaction (Blatch and Lassle, 1999), whereas the U-box is the active site of the E3 ligase (Hatakeyama *et al.*, 2001; Jiang *et al.*, 2001; Murata *et al.*, 2001). We demonstrated that, like animal CHIP proteins, AtCHIP has E3 ubiquitin ligase activity *in vitro*. Furthermore, we showed that AtCHIP plays important roles in plant growth and development, as overexpression of *AtCHIP* in Arabidopsis rendered plants more sensitive to both low and high temperatures. For example, the growth of *AtCHIP*-overexpressing plants under chilling temperature conditions is severely retarded, whereas, under high-temperature conditions, *AtCHIP*-overexpressing plants are male sterile (Yan *et al.*, 2003). It is clear that AtCHIP plays a critical role in plant cellular metabolism under temperature stress conditions.

To understand the molecular mechanism of how AtCHIP is involved in the stress response, we identified proteins that physically interact with AtCHIP by using the yeast two-hybrid technique (Luo *et al.*, 2006). Among the AtCHIP-interacting proteins identified was a nuclear-encoded component of the ATP-dependent chloroplast Clp protease. This protein, ClpP4, is a core subunit of the Clp protease complex that is probably involved in protein quality control in the chloroplast stroma (Adam and Clarke, 2002). AtCHIP interacts with ClpP4 *in vivo* and ubiquitylates ClpP4 *in vitro*, while the steady-state level of ClpP4 is reduced in *AtCHIP*-overexpressing plants under high-intensity light conditions, suggesting that the stability of ClpP4 might be directly influenced by AtCHIP ubiquitylation *in vivo*. The discovery that AtCHIP ubiquitylates ClpP4 in Arabidopsis reveals a novel regulatory mechanism for a chloroplast protease via the degradation of one of its subunit precursors in the cytosol. This finding demonstrates a direct role for the ubiquitin-mediated degradation pathway in regulating the proteolytic activity inside chloroplast.

Results

Chloroplast protein ClpP4 is a substrate protein of AtCHIP

The chloroplast proteolytic subunit ClpP4 was previously identified as an AtCHIP-interacting protein from a yeast two-hybrid screening (Luo *et al.*, 2006). To test whether ClpP4 is indeed a substrate protein of AtCHIP, we conducted an *in vitro* ubiquitylation experiment using AtCHIP as the E3 ligase, Arabidopsis UBC8 as the E2 enzyme, and ClpP4 as the substrate. As shown in Figure 1, ClpP4 could be ubiquitylated by AtCHIP *in vitro*. In the presence of all components necessary for the ubiquitylation reaction, at least three proteins of higher molecular mass were detected using a ClpP4-specific antibody (Zheng *et al.*, 2002), in addition to the expected ClpP4 polypeptide. These larger proteins corresponded in size exactly to ClpP4 with one, two or three ubiquitin molecules added, indicating that ClpP4 is indeed a substrate of AtCHIP *in vitro*.

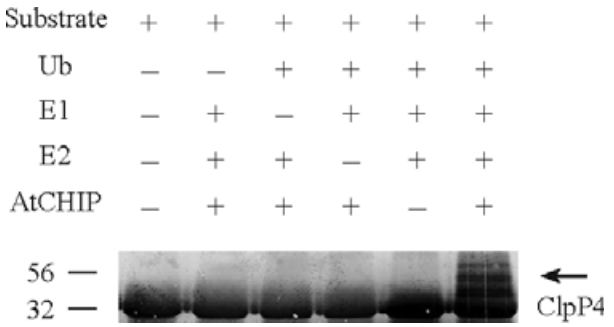


Figure 1. Ubiquitylation experiment demonstrating that AtCHIP can ubiquitylate ClpP4 *in vitro*.
The substrate used in the *in vitro* ubiquitylation reaction is ClpP4, and the antibodies used in the Western blot analysis are anti-ClpP4 antibodies. The arrow indicates ubiquitylated ClpP4 subunits.

AtCHIP overexpression reduces the steady-state level of ClpP4 under high-intensity light conditions

Because AtCHIP can ubiquitylate ClpP4 *in vitro*, the expected outcome of ClpP4 ubiquitylation *in vivo* would be degradation of ClpP4. To examine this, we analyzed the steady-state level of ClpP4 in *AtCHIP*-overexpressing plants under various growth conditions. Under normal growth and heat-shock conditions, the relative amount of ClpP4 protein was unaffected in *AtCHIP*-overexpressing plants when compared to that in wild-type plants (Figure 2a,b). Under high-intensity light condition, however, the steady-state level of ClpP4 decreased significantly in the *AtCHIP*-overexpressing plants (Figure 2c), ranging from 40% to 70% in the various transgenic lines (Figure 2d). Four *AtCHIP*-overexpressing plants, O3261, O3486, O472 and O5182, which had been shown to have at least four to fivefold more AtCHIP protein based on our previous study (Yan *et al.*, 2003), appear to have significantly reduced levels of ClpP4, indicating that there is a direct correlation between the increased level of AtCHIP and the decreased level of ClpP4 in these *AtCHIP*-overexpressing plants under high-intensity light conditions.

The transcript level of ClpP4 is not reduced in AtCHIP-overexpressing plants

The reduced levels of ClpP4 in *AtCHIP*-overexpressing plants under high-intensity light conditions could be due to reduced expression of *ClpP4* at the transcription level. In order to rule out this possibility, we conducted Northern blot

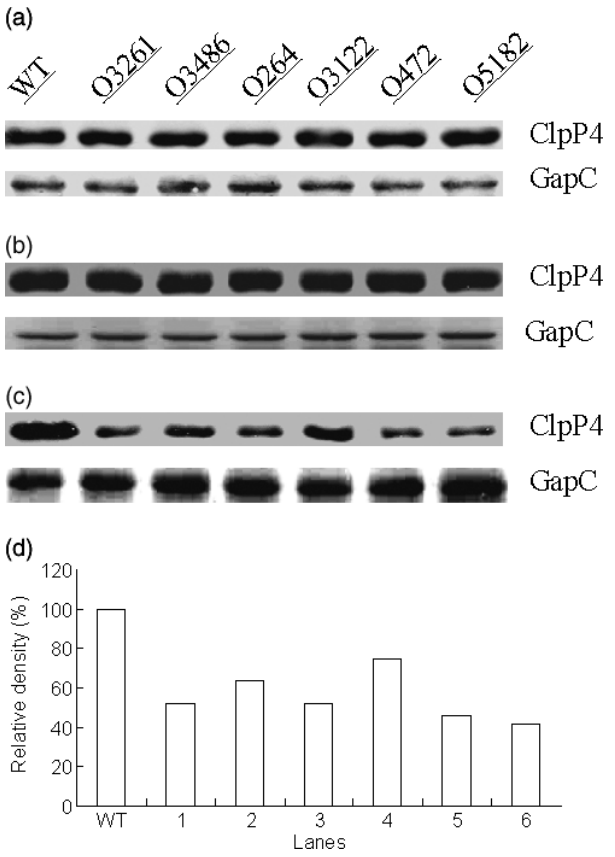


Figure 2. Steady-state levels of ClpP4 protein in *AtCHIP*-overexpressing plants under normal and stress conditions.
(a) Normal growth conditions.
(b) After heat-shock treatment.
(c) After high-intensity light treatment.
(d) Relative levels of ClpP4 in wild-type and six *AtCHIP*-overexpressing plants after high-intensity light treatment (obtained by analyzing the Western blot shown in (c) by the densitometry method). WT, wild-type; O3261, O3486, O264, O3122, O472 and O5182, six independent *AtCHIP*-overexpressing plants; lanes 1–6 in (d) correspond to lines O3261, O3486, O264, O3122, O472 and O5182, respectively; GapC, cytosolic glyceraldehyde-3-phosphate-dehydrogenase (as the loading control for Western blot).

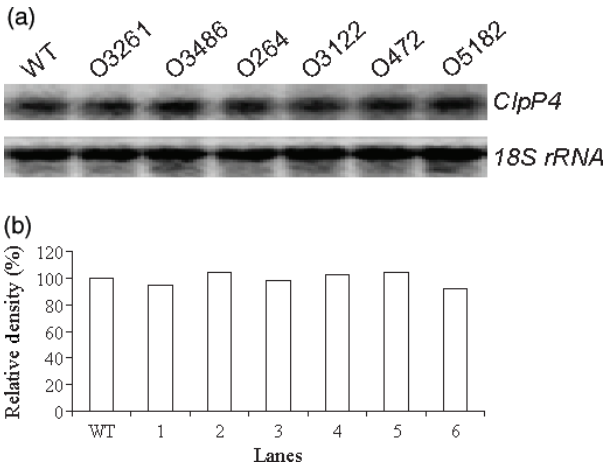


Figure 3. Analysis of *ClpP4* transcript in wild-type and *AtCHIP*-overexpressing plants after high-intensity light treatment.
(a) Total RNAs from wild-type and *AtCHIP*-overexpressing plants were hybridized to a labeled *ClpP4* cDNA fragment. The 18S rRNA was used as the RNA loading control. WT, wild-type plant; O3261, O3486, O264, O3122, O472 and O5182, six independent *AtCHIP*-overexpressing plants.
(b) Relative levels of *ClpP4* transcript in wild-type and six *AtCHIP*-overexpressing plants (obtained by analyzing the Northern blot shown in (a) by the densitometry method). Lanes 1–6 in (b) correspond to lines O3261, O3486, O264, O3122, O472 and O5182, respectively.

experiments to analyze the *ClpP4* transcript after high-intensity light treatment. Our data indicate that there are no major differences at the transcript level between wild-type and *AtCHIP*-overexpressing plants (Figure 3), suggesting that the difference observed in the protein levels between wild-type and *AtCHIP*-overexpressing plants is at the post-transcriptional level.

AtCHIP interacts with ClpP4 in vivo

If ClpP4 is a substrate of AtCHIP, these two proteins should interact with each other *in vivo*. To test this possibility, we conducted co-precipitation experiments using both wild-type and *AtCHIP*-overexpressing plants. Under normal growth conditions, AtCHIP clearly interacted with ClpP4 because we could pull down ClpP4 from leaf crude extracts by using anti-AtCHIP antibodies (Figure 4a). This interaction is very specific, because no ClpP4 could be precipitated down if no anti-AtCHIP antibodies or unrelated antibodies (e.g. anti-APX3 antibodies) were used in the co-precipitation experiments (Figure 4a). Furthermore, overexpression of *AtCHIP* only slightly increased the amount of ClpP4 precipitated (Figure 4a). Interestingly, after high-intensity light treatment, the amount of ClpP4 precipitated was significantly increased in *AtCHIP*-overexpressing plants (Figure 4b), and several proteins with higher molecular weights that are recognized by anti-ClpP4 antibodies are also increased in *AtCHIP*-overexpressing plants. These data indicate that AtCHIP does interact with ClpP4 *in vivo*, and overexpression of *AtCHIP* appears to increase their interaction *in vivo* under high-intensity light treatment.

ClpP4-overexpressing plants display chlorotic phenotypes and contain non-functional chloroplasts

In order to study the function of ClpP4 in plant cells, we fused a full-length *ClpP4* cDNA to the CaMV 35S promoter in a pBIN19-derived vector (Jefferson *et al.*, 1987), and then introduced the construct into wild-type Arabidopsis plants by using the flower-dip transformation method (Clough and Bent, 1998). We generated 33 independent transgenic plants that overexpress *ClpP4*, with most having just one T-DNA insertion based on segregation data (Table 1). Interestingly, 11 of these plants displayed a chlorotic phenotype when the

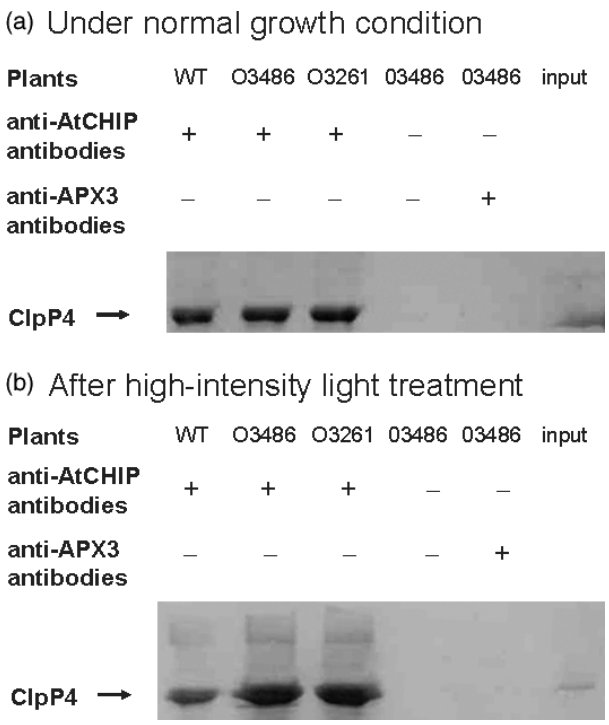


Figure 4. Co-precipitation experiments demonstrating that AtCHIP interacts with ClpP4 *in vivo*.
(a) Cellular extracts were prepared from plants grown under normal conditions. WT, wild-type plants; O3486 and O3261, two *AtCHIP*-overexpressing plants. Anti-AtCHIP antibodies were used in precipitating ClpP4 in lanes 1–3, no antibodies were used in lane 4, and anti-APX3 antibodies were used in lane 5. Lane 6 was loaded with cellular extracts directly for Western blot.
(b) Cellular extracts were prepared from plants that were treated with high-intensity light for a week.

Table 1 Segregation data and phenotype of *ClpP4*-overexpressing plants

Independent lines	Km^R	Km^S	T-DNA insertion[a]	Chlorosis[b]
WT	0	94		
CO2	88	31	1 ($P > 0.7$)	+
CO6	84	26	1 ($P > 0.7$)	+
CO20	133	44	1 ($P > 0.9$)	+
CO27	66	19	1 ($P > 0.5$)	+
CO30	62	23	1 ($P > 0.6$)	+
CO31	62	1	3 ($P > 0.9$)	+
CO32	88	26	1 ($P > 0.5$)	+
CO33	69	23	1 ($P = 1$)	+
CO53	70	23	1 ($P > 0.8$)	+
CO64	88	30	1 ($P > 0.9$)	+
CO68	76	26	1 ($P > 0.9$)	+

Km^R, kanamycin-resistant; Km^S, kanamycin-sensitive.
[a]T-DNA insertion number is based on χ^2 analysis of segregation data.
[b]The appearance of chlorosis at the rosette stage was scored as '+'.

rosette leaves were established (Figure 5). To test whether there was a correlation between *ClpP4* overexpression and chlorosis, total RNAs were extracted from wild-type and

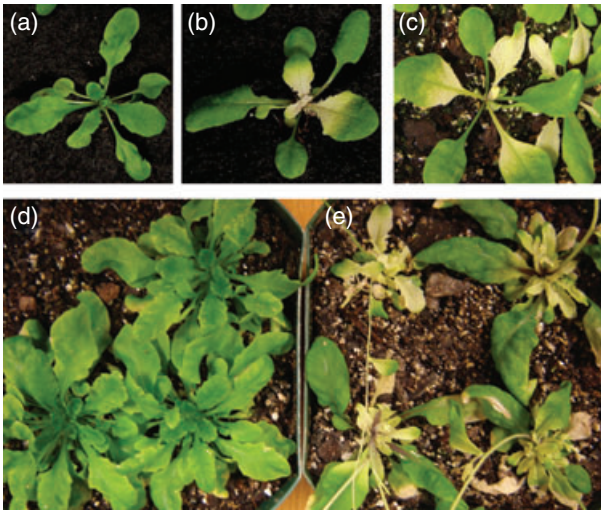


Figure 5. Phenotypes of wild-type and *ClpP4*-overexpressing plants.
(a, d) Wild-type plants; (b, c, e) *ClpP4*-overexpressing plants. Plants in (a–c) are about 4 weeks old, and plants in (d) and (e) are about 5 weeks old.

chlorotic *ClpP4*-overexpressing plants and the levels of *ClpP4* transcripts were determined by Northern blot analysis. As shown in Figure 6(a), a two to fourfold increase in *ClpP4* transcript level was observed in the various *ClpP4*-overexpressing plants. A corresponding increase in the steady-state level of ClpP4 protein was also demonstrated by Western blot analysis (Figure 6c), suggesting a causal relationship between increased expression of *ClpP4* and the chlorotic phenotype in *ClpP4*-overexpressing plants.

Other Clp proteolytic subunits are reduced in ClpP4-overexpressing plants

The proteolytic subunits of the Clp protease complex appear to exist in a structured way such that different subunits are represented in a specific stoichiometry (Peltier *et al.*, 2004), and overproduction of one subunit might disrupt the overall structure of the whole complex. We tested whether overexpression of *ClpP4* would affect the steady-state levels of other proteolytic subunits by conducting Western blot experiments, and we found that an increased level of ClpP4 did indeed lead to reduced levels of ClpP3 and ClpP5 (Figure 7). Interestingly, the level of ClpP6 was not affected. It appears that there is greater reduction in the levels of ClpP3 and ClpP5 in the chlorotic tissues than in the green tissues of chlorotic plants (Figure 7), which indicates that the loss of Clp complex might be the cause of the chlorosis.

No functional chloroplasts can be found in the chlorotic tissues of ClpP4-overexpressing plants

We next analyzed chloroplast ultrastructure in the green leaf tissues of wild-type plants and in the chlorotic tissues of

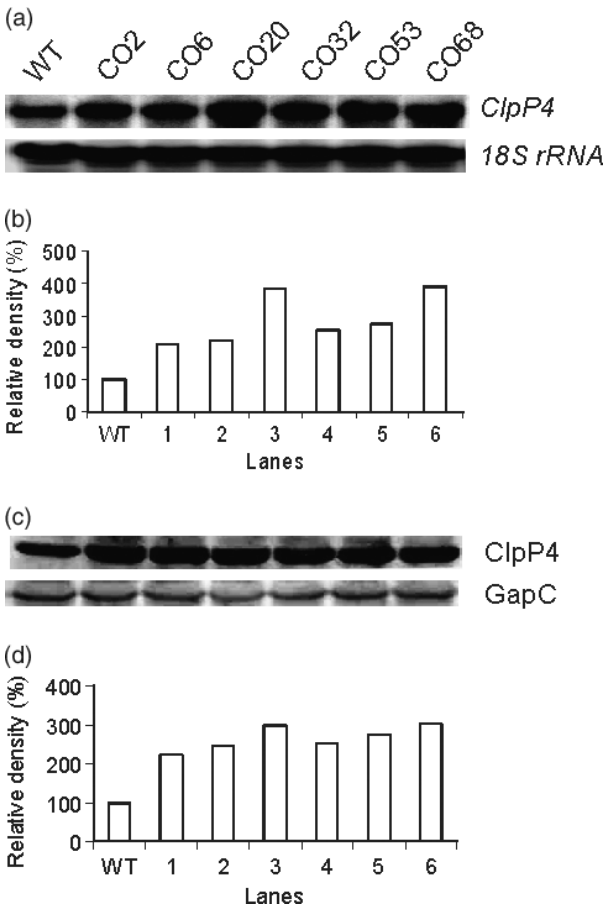


Figure 6. Steady-state levels of *ClpP4* transcript and protein in wild-type and *ClpP4*-overexpressing plants.
(a) RNA blot analysis of the transcript levels of *ClpP4* in wild-type and *ClpP4*-overexpressing plants.
(b) Relative transcript levels of *ClpP4* in wild-type and *ClpP4*-overexpressing plants (obtained by analyzing the RNA blot shown in (a) by the densitometry method).
(c) Western blot analysis of ClpP4 in wild-type and *ClpP4*-overexpressing plants.
(d) Relative levels of ClpP4 in wild-type and *ClpP4*-overexpressing plants (obtained by analyzing the Western blot shown in (c) by the densitometry method).
WT, wild-type; CO2, CO6, CO20, CO32, CO53 and CO68, six independent chlorotic *ClpP4*-overexpressing plants (lanes 1–6 in (b) and (d) correspond to these six transgenic lines, respectively). 18S rRNA was used as the loading control for Northern blot and GapC was used as the loading control for the Western blot.

ClpP4-overexpressing plants, and we observed an almost complete absence of functional chloroplast in the chlorotic tissues of the *ClpP4*-overexpressing plants (Figure 8). The chloroplasts found in the chlorotic tissues of *ClpP4*-overexpressing plants were devoid of thylakoid membranes, and were generally smaller and more spherical than those in wild-type plants, which were more reminiscent of plastids prior to chloroplast differentiation. Our data indicate that *ClpP4* overexpression interferes with normal chloroplast development and the formation of a functional thylakoid membrane network.

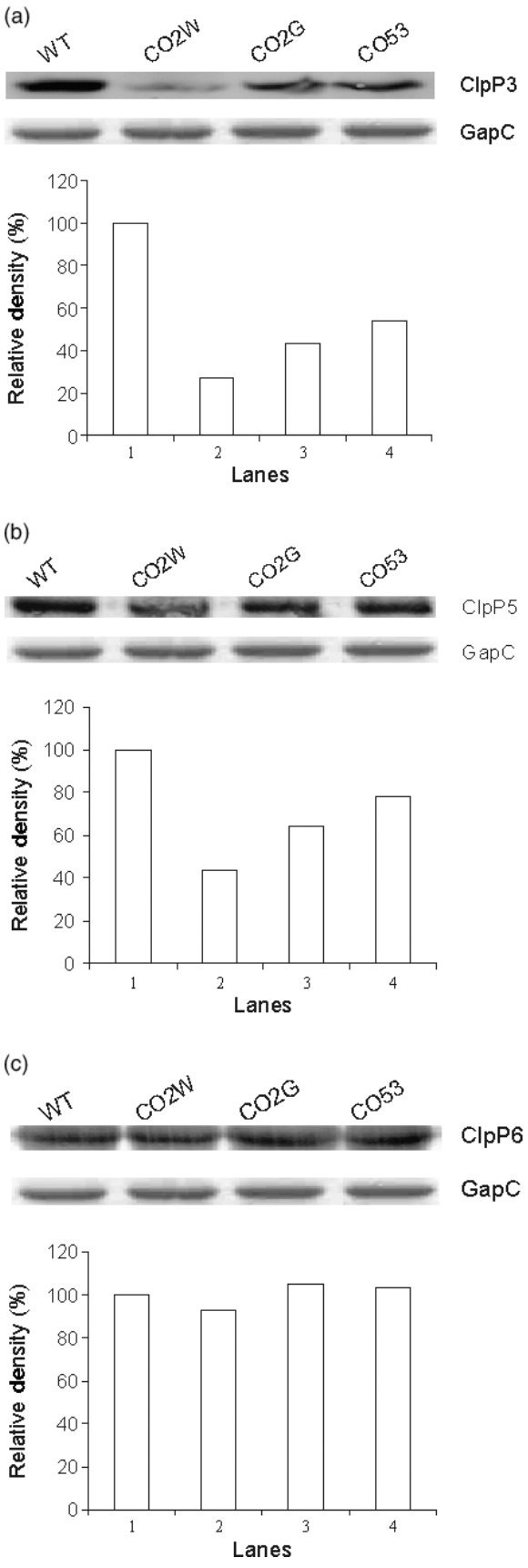
ClpP4 overexpression leads to an early-flowering phenotype

In addition to the leaf chlorosis, *ClpP4*-overexpressing plants bolted and flowered earlier than wild-type plants (Figure 9), with the phenotype being highly reproducible over four generations. As a result, it appears that an increased level of ClpP4 induces early flowering.

Discussion

ClpP4 is a proteolytic subunit of the chloroplast Clp protease in plant cells. Clp proteases are ATP-dependent enzymes that are thought to be major contributors to protein degradation in the chloroplast stroma and in mitochondria (Adam and Clarke, 2002; Clarke *et al.*, 2005; Halperin *et al.*, 2001; Zheng *et al.*, 2002), although their exact role in plants has yet to be defined (Adam and Clarke, 2002; Clarke *et al.*, 2005). In addition to the plastid-encoded ClpP1, there are five nuclear-encoded ClpP and four ClpP-like paralogs in Arabidopsis, all of which are located in chloroplasts except for ClpP2, which is targeted to mitochondria (Halperin *et al.*, 2001; Peltier *et al.*, 2004). To date, all chloroplast ClpP proteins identified have been found to be essential for plant development (Clarke *et al.*, 2005; Kuroda and Maliga, 2003). All chloroplast ClpP and ClpR paralogs associate within a single proteolytic core complex of approximately 325 kDa (Peltier *et al.*, 2004), presumably in a barrel-like structure of two heptameric rings forming a single proteolytic cavity in analogy to the model Clp protease in *Escherichia coli* (Wang *et al.*, 1997). The Clp proteolytic core depends on its cognate apparatus, which consists of a single hexameric ring of a chaperone regulatory subunit, ClpC in the case of chloroplasts. The overall structure of the Clp protease resembles that of the 26S proteasome in eukaryotes (Horwich *et al.*, 1999; Wickner *et al.*, 1999). Our data indicate that eukaryotic CHIPs not only physically bind to proteasomes (Connell *et al.*, 2001; Demand *et al.*, 2001; Meacham *et al.*, 2001) but may also bind to a component of the Clp protease complex that is a major part of the protein degradation machinery in chloroplasts.

One of the major findings in this study is the discovery that AtCHIP ubiquitylates ClpP4 *in vitro* (Figure 1). Together with the observations that *AtCHIP* overexpression leads to a reduced steady-state level of ClpP4 under high-intensity light conditions (Figure 2) and that AtCHIP and ClpP4 interact with each other *in vivo* (Figure 4), AtCHIP appears to be involved in a ubiquitylation-related process that helps to regulate Clp proteolytic activity in chloroplasts. Under normal growth conditions, *AtCHIP* overexpression does not lead to ClpP4 degradation, which suggests that other factor(s) might be required for initiating ClpP4 degradation under high-intensity light conditions. These other factor(s)



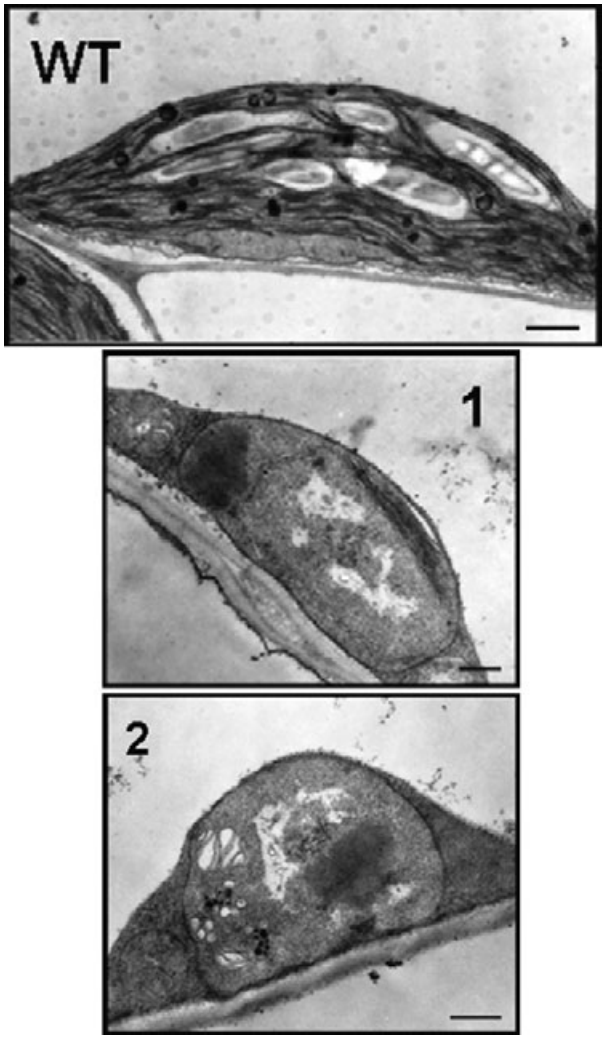


Figure 8. Chloroplast structures in wild-type (WT) and *ClpP4*-overexpressing chlorotic leaves (1 and 2). Bars = 0.5 μm.

may include some stress-inducible E2 enzymes or proteins that increase AtCHIP and ClpP4 interaction under high-intensity light conditions (Figure 4b), and which eventually lead to increased ClpP4 ubiquitylation and degradation in *AtCHIP*-overexpressing plants. Although ClpP4 appears to be a substrate protein of AtCHIP *in vivo*, one could argue that

Figure 7. Steady-state levels of ClpP3, ClpP5 and ClpP6 in wild-type and *ClpP4*-overexpressing plants.
(a) ClpP3 and its level relative to GapC in wild-type and *ClpP4*-overexpressing plants.
(b) ClpP5 and its level relative to GapC in wild-type and *ClpP4*-overexpressing plants.
(c) ClpP6 and its level relative to GapC in wild-type and *ClpP4*-overexpressing plants.
WT, wild-type; CO2W, chlorotic tissues from *ClpP4*-overexpressing line CO2; CO2G, green tissues from *ClpP4*-overexpressing line CO2; CO53, chlorotic and green tissues (about 80% and 20%, respectively) from *ClpP4*-overexpressing line CO53. GapC was used as the loading control for the Western blot.

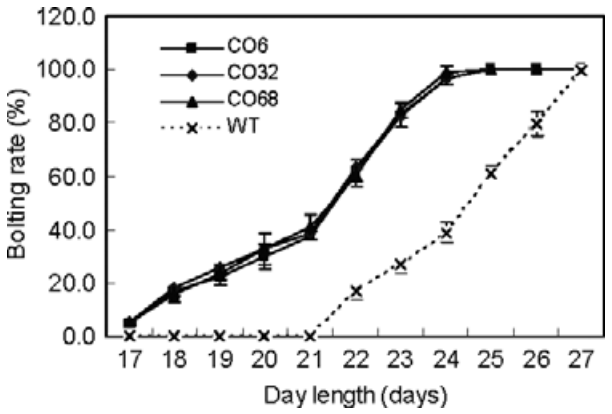


Figure 9. The timing of bolting is shortened in *ClpP4*-overexpressing plants. WT, wild-type plants; CO6, CO32 and CO68, three independent *ClpP4*-overexpressing plants. Error bars represent SD, $n = 48$.

an E3 enzyme should only recognize non-native or damaged proteins, not those proteins detected by the two-hybrid technique in yeast cells. In fact, there is no evidence that E3 enzymes only recognize non-native proteins. On the contrary, ubiquitylation does occur on native proteins, and these ubiquitylated proteins may have altered cellular destination or enhanced activity (Pickart, 2001; Wojcik, 2001).

In this study, we further analyzed the function of *ClpP4* in Arabidopsis. We created gain-of-function mutants by overexpressing *ClpP4* in transgenic plants, and we obtained a chlorotic phenotype (Figure 5) similar to that found in some *ClpP4* antisense plants described by Zheng *et al.* (2007). The chlorotic phenotype observed in *ClpP4*-overexpressing plants is probably caused by increased levels of ClpP4 protein in transgenic plants, because the steady-state levels of ClpP4 are clearly higher in transgenic plants than that in wild-type plants (Figure 6). Examination of cellular structures of transgenic plants reveals that no functional chloroplasts could be found in the chlorotic tissues (Figure 8), which explains why these transgenic plants become chlorotic. Our data from analyzing *ClpP4*-overexpressing plants, together with the data from analyzing *ClpP4* antisense plants (Zheng *et al.*, 2006), support the notion that the Clp proteolytic subunits are represented in certain ratios in the Clp complex, and overproduction or under-production of one subunit may disrupt the overall structure of Clp complex, which might lead to degradation of other unassembled subunits in the chloroplast. It appears that the more closely related protein ClpP3 is reduced more than ClpP5, and the least related ClpP6 is almost unaffected in *ClpP4*-overexpressing plants (Figure 7), suggesting that there might be direct competition between closely related Clp proteolytic subunits in forming the Clp complex, and that Clp complexes in which the Clp4 subunit is over-represented is not functional in chloroplasts.

Nuclear-encoded chloroplast proteins are imported into chloroplasts in an unfolded conformation (Schatz and Dobberstein, 1996), and, during this process, two cytosolic Hsp70 chaperones are probably involved in maintaining unfolded precursors in a transport-competent state (Caliebe and Soll, 1999; Fuks and Schnell, 1997; Keegstra and Cline, 1999). As CHIP was initially identified as a chaperone co-factor that negatively regulates Hsp70 (Ballinger *et al.*, 1999), and CHIP can target substrates of Hsp70 to the 26S proteasome for degradation (Höhfeld *et al.*, 2001; Murata *et al.*, 2001), it would not be surprising if AtCHIP competes with Hsp70 for substrate proteins such as the ClpP4 precursor in the cytoplasm prior to its import into the chloroplast, especially when increased demand for protein import into chloroplasts is impaired under certain stress conditions. Indeed, many nuclear-encoded chloroplast proteins were identified from the yeast two-hybrid screening as possible substrates of AtCHIP, such as the small subunit of Rubisco and the chlorophyll *a*/*b*-binding proteins (e.g. RbcS-3b and Lhcb6, respectively; Luo *et al.*, 2006). The specific targeting of the ClpP4 precursor for ubiquitylation and degradation by AtCHIP, however, presents a cytosolic control over the amount of ClpP4 precursor available for chloroplast import. Controlling the amount of ClpP4 imported into chloroplasts would affect the amount of functional Clp proteolytic core complex in the stroma. We have recently shown that down-regulation of a single ClpP paralog proportionally reduces the amount of total Clp proteolytic core and thereby functional Clp protease (Sjögren and Clarke, unpublished data).

Protein degradation via the ubiquitin–proteasome system has been extensively studied over the years, and there is a wealth of knowledge regarding the protein degradation system and its involvement in many important cellular processes in eukaryotic cells (Ciechanover *et al.*, 2000; Ferrell *et al.*, 2000; Smalle and Vierstra, 2004). In contrast, little is known about protein degradation in organelles. Only recently have the identity and abundance of the major proteases within chloroplasts been revealed, all of which are of bacterial origin (Adam and Clarke, 2002). Despite this, essentially nothing is known about how the various proteolytic activities within plant organelles such as the chloroplast are regulated. Our discovery that AtCHIP controls protein degradation in the chloroplast improves our understanding of protein quality control in chloroplasts. Animal CHIP proteins have been shown to be critical in protein quality control in the cytoplasm of animal cells (Cyr *et al.*, 2002; Murata *et al.*, 2001; Patterson, 2002), yet our data indicate that AtCHIP may be important in protein degradation metabolism in the chloroplast, hinting that CHIP is probably a regulator that coordinates protein degradation metabolism in both

cytoplasm and organelles. As AtCHIP is an U-box-containing E3 ligase, an understanding of the role of AtCHIP in protein quality control in both cytoplasm and chloroplast as well as in the stress response will contribute significantly to our understanding of protein homeostasis that is central to plant growth and development under normal and stress conditions.

Experimental procedures

Identification of ClpP4 as an AtCHIP-interacting protein

ClpP4 was identified as one of the AtCHIP-interacting proteins from a yeast two-hybrid screening (Luo *et al.*, 2006). The nuclear gene coding for ClpP4 was previously described by Adam *et al.* (2001) and Zheng *et al.* (2002), and was given the gene identification number At5g45390.

Expression of ClpP4 *in bacterial cells and* in vitro *ubiquitylation of ClpP4*

The full-length *ClpP4* gene was amplified from an Arabidopsis cDNA library with the primers Clp-1 (5′-GTCGGAATTCCATGGGAACCCTATCTCTCT-3′) and Clp-2 (5′-GTCGGAGCTCTTAGTAGATCTCATCATCAGGT-3′), and then cloned into the pET-30b vector (Novagen, Madison, WI, USA) using restriction enzymes *Eco*RI and *Sac*I. Recombinant vectors were introduced in the bacterial strain BL21(DE3) for expression in the presence of kanamycin. ClpP4 was purified according to the manufacturer's protocol (His-Bind Kits, Novagen, Madison, WI, USA). The *in vitro* ubiquitylation reaction was conducted as described previously (Yan *et al.*, 2003), except that the reaction mixture includes AtCHIP as the E3 ligase, AtUBC8 as E2 conjugase, rabbit E1 (Calbiochem, San Diego, CA, USA) as activase, and bovine ubiquitin (Sigma, St. Louis, MO, USA). Polyclonal antibodies against ClpP4 (Zheng *et al.*, 2002) were used in the Western blot as shown in Figure 1.

Construction of transforming vector for overexpressing ClpP4 *in Arabidopsis*

The full-length *ClpP4* cDNA was amplified from a cDNA library with the primers Clp-3 (5′-GTCGTCTAGAATGGGAACCCTATCTCTCTC-3′) and Clp-2, digested with enzymes *Xba*I and *Sac*I, and then subcloned into the pBI121 vector (Jefferson *et al.*, 1987) by replacing the GUS gene, to form a transforming vector that was transformed into the *Agrobacterium tumefaciens* GV3101 and confirmed by PCR. The Arabidopsis transformation was conducted according to the protocol of Clough and Bent (1998).

Co-precipitation experiments

Leaf proteins were extracted by grinding leaves in liquid nitrogen in extraction buffer (50 mM $NaPO_4$, pH 7.0, 1 mM EDTA). The crude extracts were centrifuged in a microfuge at 13 000 ***g*** for 10 min, and the protein concentration in supernatants was determined by the Bradford (1976) method using bovine serum albumin as a standard. About 200 μl of supernatant (containing 500 μg of total proteins) were incubated with 15 μl of anti-AtCHIP antibodies at 4°C for 1 h, and then 50 μl of Protein A–agarose (Sigma catalog number P0932) were added, and the mixture was incubated for an additional 3 h. The Protein A–agarose complex was washed five times with the extraction buffer before adding an equal volume of 2x SDS loading buffer (125 mM Tris-Cl, 2% SDS, 20% glycerol, 200 mM dithiothreitol, 0.01% bromophenol blue, pH 6.8). Samples were boiled at 100°C for 5 min before loading for electrophoresis in a 12% SDS polyacrylamide gel. The conditions for blotting and color development were the same as described previously (Yan *et al.*, 2003), and the antibodies used were anti-ClpP4 antibodies.

Plant growth and stress treatments

Arabidopsis seeds (ecotype C24) were surface-sterilized in 75% ethanol for 1 min, followed by soaking in 50% bleach (Clorox, Oakland, CA, USA) for 10 min, and then rinsed extensively in sterile water. Plant seeds were sown on MS agar plates (Murashige and Skoog, 1962) and stored for 4 days at 4°C before being moved to 25°C under continuous white light conditions (150 μmol m^{-2} sec^{-1}). For bolting timing measurement, seedlings of similar size were then transplanted into soil and allowed to grow until flowering. The time of bolting was scored as the time that the inflorescence stalk emerges from the apical meristem. The data represent the means ± SD from 48 plants, and the experiment was repeated three times. For stress treatments, seedlings were then transplanted into soil and allowed to grow for 3 weeks before stresses were applied. The heat treatment consisted of 21 h at 25°C and 3 h at 42°C per day for a week. The high-intensity light treatment consisted of 18 h under normal light (150 μmol m^{-2} sec^{-1}) and 6 h under high-intensity light (1500 μmol m^{-2} sec^{-1}) per day for a week.

RNA isolation and hybridization

Total RNAs were isolated from Arabidopsis plants using TRIZOL reagent (Invitrogen, Carlsbad, CA, USA), separated by electrophoresis (10 μg per lane), blotted to a nylon membrane, and hybridized with various probes. Hybridization was carried out according to the method described by Church and Gilbert (1984) using probes labeled by random priming. The washing conditions were as follows: twice (10 min each) in a solution of 0.5% BSA, 1 mM EDTA, 40 mM $NaHPO_4$ (pH 7.2) and 5.0% SDS at 63°C, then four times (5 min each) in a solution of 1 mM EDTA, 40 mM $NaHPO_4$ (pH 7.2) and 1% SDS at 63°C. The same filter was used for hybridizations with probes *ClpP4* and *18SrRNA*, consecutively. The conditions for stripping the filter were as follows: twice (15 min each) in 2 mM Tris (pH 8.2), 2 mM EDTA (pH 8.0) and 0.1% SDS.

Immunoblot analysis

Leaf proteins were extracted by grinding mature leaves in a mortar in extraction buffer (50 mM $NaPO_4$, pH 7.0, 1 mM EDTA). The crude extracts were centrifuged in a microfuge at 13 000 ***g*** for 10 min, and the supernatants, which contain mainly soluble proteins, were added to an equal volume of 2 x SDS loading buffer (125 mM Tris-Cl, 2% SDS, 20% glycerol, 200 mM dithiothreitol, 0.01% bromophenol blue, pH 6.8). Protein concentration in the extraction buffer was determined by the Bradford (1976) method using bovine serum albumin as a standard. Proteins from transgenic plants and wild-type plants were subjected to electrophoresis in a 12% SDS polyacrylamide gel. The conditions for blotting and color development were the same as described previously (Yan *et al.*, 2003), except the antibodies used were anti-ClpP3, anti-ClpP4, anti-ClpP5, anti-ClpP6 and anti-GapC antibodies.

Electron microscopic analysis

Three- to four-week-old Arabidopsis plants were harvested and immediately fixed in 2% glutaraldehyde in 0.2 M phosphate (pH 7.2) for 8 h at room temperature, and post-fixed overnight at room temperature with 0.5% osmium tetroxide in the same buffer. Tissues were dehydrated with an ethanol series and embedded in an epoxy resin. Thin sections were stained with uranyl acetate and lead citrate and examined with a JEOL 1200EX electron microscope at 80 kV (JEOL, Tokyo, Japan).

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