Journal of Experimental Botany Advance Access published June 17, 2015

Journal of Experimental Botany
doi:10.1093/jxb/erv286

RESEARCH PAPER

The E3 ligase AtCHIP positively regulates Clp proteolytic subunit homeostasis

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Received 20 January 2015; Revised 7 May 2015; Accepted 13 May 2015

Editor: Christine Raines

Abstract

The caseinolytic peptidase (Clp) core proteins are essential for plant growth and development, especially for chloroplast function. Antisense or overexpression of *ClpP4,* which is one of the Clp core subunits, causes chlorotic phenotypes in *Arabidopsis*. An E3 ligase gene, *AtCHIP*, has previously been found to ubiquitylate ClpP4 *in vitro*. *ClpP4* antisense and overexpressing plants that also overexpressed *AtCHIP* were constructed to explore the effect of AtCHIP on ClpP4. Overexpression of *AtCHIP* was found to rescue the chlorotic phenotypes of both *ClpP4* antisense and overexpressing plants. The unbalanced levels of Clp core proteins in *ClpP4* antisense and overexpressing plants with overexpression of *AtCHIP* were similar to wild-type levels, suggesting that AtCHIP regulates Clp core proteins. The results also show that AtCHIP can interact with ClpP3 and ClpP5 in yeast and ubiquitylate ClpP3 and ClpP5 *in vitro*. This suggests that AtCHIP is directly related to ClpP3 and ClpP5. Given these results, the inference is that through selective degradation of Clp subunits, *AtCHIP* could positively regulate homeostasis of Clp proteolytic subunits and maximize the production of functional chloroplasts. Similar results were obtained from transgenic tobacco plants, suggesting that regulation of the Clp protease by AtCHIP is conserved.

Key words: AtCHIP; chloroplast protease; Clp proteolytic subunits; ClpP4; ClpP3; ClpP5; protein homeostasis.

Introduction

Caseinolytic protease (Clp) is a major protease system in plant cells and functions to remove mis-folded, damaged, short-lived regulatory, and otherwise unneeded proteins in plastids to maintain protein homeostasis (Adam and Clarke, 2002; Olinares *et al.*, 2011*b*). The Clp complex contains two heptameric rings as its catalytic core, the P-ring and the R-ring, with catalytic sites arranged inside the cylindrical inner core, preventing inadvertent protein degradation in the plastid (Clarke, 2012). The P-ring contains four Clp catalytic proteins, ClpP3, ClpP4, ClpP5, and ClpP6, in a ratio of 1:2:3:1. The R-ring contains one catalytic protein, ClpP1, and four non-catalytic Clp proteins, ClpR1, ClpR2, ClpR3, and ClpR4, in a ratio of 3:1:1:1:1 (Kress *et al.*, 2009; Olinares *et al.*, 2011*a*). Other than the Clp enzymatic core, a hexameric ring-like structure that is made of the ATP-dependent chaperones ClpC1, ClpC2, and ClpD is attached to the Clp

core. It is responsible for selecting, unfolding, and feeding the substrate proteins into the cavity of the enzymatic core of Clp for digestion (Yu and Houry, 2007; Stanne *et al.*, 2009; Clarke, 2012; Tryggvesson *et al.*, 2012). Two additional regulatory proteins, ClpT1 and ClpT2, appear to play roles in facilitating the interaction between the Clp core complex and the hexameric ring of chaperones, thereby assembling the Clp protease in plastids (Kato and Sakamoto, 2010; Sjogren and Clarke, 2011). Among the Clp subunit proteins in higher plants, ClpP1 is the only protein that is encoded by the chloroplast genome; the rest are all encoded by the nuclear genome and are post-translationally inserted into plastids (Adam and Clarke, 2002; Bryant *et al.*, 2011; Olinares *et al.*, 2011*b*). ClpP2 is targeted to mitochondria, where it forms a homomeric tetradecameric protein core that is similar to the Clp proteases in bacterial systems (Peltier *et al.*, 2004). In contrast, the nine Clp subunit proteins ClpP1, ClpP3–P6, and ClpR1–R4 form a very complex tetradecameric protein core (P-ring + R-ring), making Clp protease in plastids the most complex Clp protease system in any living organism (Clarke, 2012; Olinares *et al.*, 2011*b*).

The functions of many Clp core subunit proteins have been studied in detail in the last 10 years, and except for one Clp subunit protein, ClpP1, all of the other Clp core proteins are essential for plant growth and development, especially for chloroplast function (Adam and Clarke, 2002; Olinares *et al.*, 2011*b*; Kim *et al.*, 2013). ClpP1 is essential for leaf development in *Nicotiana tabacum* (tobacco) plants (Shikanai *et al.*, 2001; Kuroda and Maliga, 2003). ClpP3 is not as important as ClpP4 and ClpP5, because the knockout mutant for ClpP3, *clpp3-1*, can grow beyond the cotyledon stage under heterotrophic conditions (i.e. with the addition of sucrose in the media), whereas the complete loss of ClpP4 and ClpP5 is embryo lethal (Kim *et al.*, 2009; Kim *et al.*, 2013). The more important role of ClpP4 and ClpP5 compared to ClpP3 is likely due to the fact that both ClpP4 and ClpP5 are represented in multiple copies in the Clp core complex (Derrien *et al.*, 2012; Dong *et al.*, 2013; Kim *et al.*, 2013). The antisense repression of *ClpP6* led to a variegated or chlorotic phenotype (Sjogren *et al.*, 2006; Zheng *et al.*, 2006), suggesting that ClpP6 also plays an important role in chloroplast development and function. Among the four non-catalytic ClpR proteins, ClpR1 is likely the least important Clp subunit protein because its knockout mutant *clpr1-1* could be maintained in soil and produce seeds (Koussevitzky *et al.*, 2007; Zybailov *et al.*, 2009). The overexpression of *ClpR3* in the *clpr1-1* mutant could rescue the phenotype of *clpr1-1*, whereas the overexpression of *ClpR2* and *ClpR4* in *clpr1-1* could not, indicating that there is functional redundancy between ClpR1 and ClpR3 (Kim *et al.*, 2009). The loss of ClpR2 and ClpR4 both led to delayed embryogenesis and developmental arrest at the cotyledon stage, indicating that like other ClpP subunits in the chloroplast (Rudella *et al.*, 2006), these ClpR subunits also play critical roles in the overall function of the Clp protease in plastids.

Animal CHIP proteins are E3 ubiquitin ligases that interact with Hsp70 and Hsp90 and target their substrate proteins to the 26S proteasome for degradation (Ballinger *et al.*, 1999). Like the animal CHIPs, *Arabidopsis thaliana* CHIP have also been found to interact with cytosolic Hsp70 (Shen *et al.*, 2007*a*). However, *AtCHIP* overexpression plants were more sensitive to temperature stress and the plant hormone abscisic acid (Yan *et al.*, 2003; Luo *et al.*, 2006). This was unlike the animal CHIP, for which the overexpression led to increased stress tolerance and recovery ability in animal cells (Dai *et al.*, 2003). The first two interactors identified for AtCHIP were two regulatory subunits of the type 2A protein phosphatise (PP2A), PP2AA3, and reticulocalbin 1 (RCN1)/PP2AA1. The overexpression of *AtCHIP* conferred increased PP2A activity and abscisic acid sensitivity in plants, which was opposite to *RCN1* overexpressing plants (Deruère *et al.*, 1999; Kwak *et al.*, 2002; Luo *et al.*, 2006). Another two target proteins of AtCHIP were ClpP4 and FtsH1 (Shen *et al.*, 2007*a*; Shen *et al.*, 2007*b*). AtCHIP has been suggested to induce the degradation of ClpP4 and therefore down-regulate the Clp protease under high-intensity light conditions (Shen *et al.*, 2007*b*). The degradation of FtsH1 was also promoted by AtCHIP and the activity of FtsH protease was decreased in *AtCHIP* overexpressing plants under high-intensity light conditions (Shen *et al.*, 2007*a*). ClpP4 and FtsH1 are two proteins located in the chloroplast, and AtCHIP has been found to interact with their cytosolic precursors to regulate the activity of Clp and FtsH protease. These results revealed a novel role for AtCHIP in regulating protein degradations in chloroplast (Yee and Goring, 2009).

Previous studies have demonstrated that Clp core complex subunits ClpP3, ClpP4, ClpP5, and ClpP6 present in a 1:2:3:1 ratio in wild-type *Arabidopsis* and that there are correlations among Clp subunit proteins, i.e. overaccumulation of ClpP1, ClpP5, ClpP6, and ClpR3 was observed in a ClpP3 null mutant and overexpression of ClpP4 has been reported to lead to reduced levels of ClpP3 and ClpP5 (Shen *et al.*, 2007*b*; Kim *et al.*, 2013). However, the mechanism behind these correlations remains unclear. In the present study, AtCHIP has been found to play an important role in the homeostasis of the Clp core subunit proteins ClpP3, ClpP4, and ClpP5 by selective ubiquitin-dependent proteolysis. The results can help us understand at least parts of the upstream mechanism of the subunit-dependent Clp protease complex.

Materials and methods

Construction of the transforming vector

The plant transformation vector PBI 121 was used to prepare the *ClpP4* antisense construct. The *ClpP4* cDNA was amplified from an *Arabidopsis* cDNA library with primers Clp-5 and Clp-6. After digestion with the restriction enzymes SacI and XbaI, the full-length *ClpP4* cDNA was ligated into the vector in reverse orientation by replacing the *GUS* gene (Jefferson *et al.*, 1987). The *AtCHIP*-overexpression construct was the same one that was used in a previous report (Yan *et al.*, 2003). The PCR primer sequences used were Clp-5: GTCGGAGCTCATGGGAACCCTATCTCTCTC; and Clp-6: GTCGTCTAGATTAGTAGATCTCATCATCAGGT.

Arabidopsis *and* Nicotiana *transformation*

The *Agrobacterium tumefaciens* strain GV3101 was used to transform *A. thaliana* ecotype Columbia-0 and *N. tabacum* ecotype SR-1 plants. The *Arabidopsis* was transformed by the floral dip method

(Clough and Bent, 1998), and the tobacco transformation was conducted according to Horsch *et al.* (1985).

Plant material and growth conditions

Thirty-eight independent *ClpP4* antisense *Arabidopsis* plants were created, most of which appeared to have just one T-DNA insertion based on segregation analysis among the T_2 progenies. *ClpP4*-overexpressing plants (CO) were constructed in a previous study (Shen *et al.*, 2007*b*). Twenty-one independent *ClpP4* antisense tobacco lines (Tca), 17 independent *ClpP4*-overexpressing tobacco lines (TCO), and 19 independent *AtCHIP*-overexpressing tobacco lines (TAO) were created. One homozygous line carrying a single insertion for Tca and one for TCO, which were both fertile and severely chlorotic, were chosen for further studies. The double transgenic tobacco lines were generated by crossing Tca (♀) or TCO (♀) to TAO (♂), respectively. One of the Tca × TAO and one of the TCO × TAO F_1 plants showing chlorotic phenotypes (♂) were then chosen to backcross to the female parental line Tca (♀) or TCO (♀), respectively. Double transgenic plants were obtained by backcrossing for three generations. For each generation, the overexpression of *AtCHIP* was confirmed by PCR analysis. The plants with overexpression of *AtCHIP* in the Tca background were named TcaA, while those in TCO background were named TCOA. After confirmation by DNA and RNA blot analysis, the TcaA and TCOA plants were used for subsequent analysis.

For *Arabidopsis* growth, seeds were surface-sterilized in 75% ethanol for 30 s and then soaked in 10% sodium hypochlorite for 3 min, followed by washing with sterile water five times. Plant seeds were then placed on Murashige-Skoog agar plates and stratified for 3 days at 4°C before being moved to 21°C under a photoperiod of 16 h light/8 h dark. For tobacco plant growth, seeds were sown on 50 × 80 cm trays and grown in a greenhouse at 26 ± 2°C.

DNA blot analysis

Genomic DNA was isolated from leaves of wild-type, Tca, TcaA, TCO, and TCOA tobacco plants and digested with the restriction enzymes XbaI and BamHI. The hybridization was then carried out according to a previous report (Pasapula *et al.*, 2011).

RNA blot analysis

Total RNA was isolated from *Arabidopsis* and tobacco leaves using the TRIzol reagent according to the manufacturer's instruction (Takara Bio, Japan). The experimental conditions for RNA blot analysis were the same as those described in Yan *et al.* (2003) and Shen *et al.* (2007*b*).

Western blot analysis

Leaf protein extracts were prepared by grinding mature leaves with extraction buffer (50 mM Tris-HCL, pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.1% Triton X-100, 0.2% NP-40, 0.6 mM PMSF, 20 μM MG132, and 100 μM protease inhibitor cocktail). The samples were mixed and put on ice for 30 min and then centrifuged for 10 min at 13 000 g at 4°C. The supernatants containing total proteins were used for the experiments. The protein concentration in the extraction buffer was determined by the bicinchoninic acid assay, and bovine serum albumin was used as a standard. For immunoblots, the protein extractions were mixed with an equal volume of 2× SDS loading buffer (125 mM Tris-Cl, 2% SDS, 20% glycerol, 200 mM dithiothreitol, and 0.01% bromophenol blue, pH 6.8) and subjected to electrophoresis in a 12% SDS polyacrylamide gel. The conditions for blotting were as previously described (Yan *et al.*, 2003). Polyclonal antibodies for AtCHIP were constructed by Yan *et al.* (2003), while anti-ClpP3, anti-ClpP4, anti-ClpP5, and anti-ClpP6 (Zheng *et al.*, 2002) were used in the western blots (Shen *et al.*, 2007*b*). Antibodies against other chloroplast proteins and the secondary antibodies that were used in this study were obtained from Sigma-Aldrich (St Louis, MO, USA).

Yeast two-hybrid assay

For the yeast two-hybrid assay, the full-length cDNAs of *AtCHIP* and *ClpP1–6* were amplified from an *Arabidopsis* cDNA library. For *AtCHIP*, *ClpP1*, *ClpP3*, *ClpP4*, and *ClpP6*, cDNAs were digested with the restriction enzymes *Eco* RI and *Xho* I. *AtCHIP* was inserted into the bait vector pEG202, and *ClpP1*, *ClpP3*, *ClpP4*, and *ClpP6* were inserted into the prey vector pJG4-5. For *ClpP2* and *ClpP5*, the cloned cDNAs were inserted into pJG4-5 using the In-fusion PCR cloning system (Clontech, USA). The protocol for analysing protein–protein interactions in the yeast two-hybrid system was described by Golemis *et al.* (2001; Shen *et al.*, 2010). HM1-1 was used as an unrelated protein control. The DNA sequences of the primers that were used for cloning can be found in Supplementary Table S1.

Expression of ClpP3 *and* ClpP5 *in bacterial cells and* in vitro *ubiquitylation experiments*

The full-length *ClpP3* and *ClpP5* gene was amplified from the *Arabidopsis* cDNA library, and the primers that were used for *ClpP3* cloning were ClpP3-1 and ClpP3-2 and for *ClpP5* cloning were ClpP5B-1 (GTCGGGATCCATGGCTCATGCTTGCGTCTC) and ClpP5-2 (Supplementary Table S1). The amplified cDNAs were cloned into the pET-30b vector (Novagen, Madison, WI, USA) using the restriction enzymes EcoRI and XhoI (ClpP3) or BamHI and XhoI (ClpP5). The recombinant vectors were expressed in BL21(DE3), and bacterially expressed ClpP3 and ClpP5 were purified according to the manufacturer's protocol (His-Bind Kits, Novagen). The *in vitro* ubiquitylation assay was conducted as previously described by Shen *et al.* (2007*b*). The ubiquitylation reaction mixture included AtCHIP as the E3 ligase, AtUBC8 as the E2 conjugase, rabbit E1 as an activase, and bovine ubiquitin, ClpP3, or ClpP5 as a substrate. Polyclonal antibodies against ClpP3 and ClpP5 were used in the western blot.

Electron microscopic analysis

To observe the chloroplast structures, the leaves from 3- to 4-week-old *Arabidopsis* plants were harvested and immediately fixed for 8 h in 0.2 M phosphate (pH 7.2) buffer containing 2% glutaraldehyde (w/v) at room temperature. After being rinsed three times in 0.2 M phosphate (pH 7.2) buffer, the tissues were post-fixed overnight in 0.5% osmium tetroxide in fixative solution. The tissues were dehydrated in an ethanol series of 75% and 90%, and twice at 100%, and embedded in an epoxy resin. Thin sections were stained with uranyl acetate and lead citrate. The sections that were used for electron microscopy were examined with a JEOL 1200EX electron microscope at 80 kV.

Leaf gas exchange measurements

The leaf gas exchange measurements were taken with *Arabidopsis* and tobacco plants using a portable photosynthesis system (LI-COR 6400; LI-COR Inc., Lincoln, NE, USA). Young, fully expanded, intact leaves of 3- to 4-week-old plants were used for the gas exchange measurements. The leaf net CO_2 assimilation rate, stomatal conductance, and transpiration were determined with the LI-COR 6400 setting at 400 μmol mol^{-1} CO_2 concentration, 60% relative humidity, 25°C chamber temperatures, 500 μmol s^{-1} air flow, and a light intensity of 1500 μmol m^{-2} s^{-1}. Five replicates were taken for each sample.

Statistical analysis

The SPSS package program version 16.0 (SPSS Inc. Chicago, IL, USA) was used to perform the statistical analysis. The data were analysed by a one-way ANOVA model. The means were compared

using the least significant difference (LSD) test at a 95% confidence level ($P < 0.05$). The values were reported as means with the standard error for all results.

Results

Both Arabidopsis ClpP4 *antisense and overexpressing plants displayed chlorotic phenotypes*

Zheng *et al.* (2006) reported that the down-regulation of *ClpP4* in *Arabidopsis* using antisense techniques led to a chlorotic phenotype; this discovery was very interesting because the overexpression of *ClpP4* gave rise to a similar phenotype (Shen *et al.*, 2007*b*). *ClpP4* antisense (ca) plants were also created to compare and analyse these two different transgenic populations in *Arabidopsis*. It was previously demonstrated that *ClpP4*-overexpressing plants (CO) displayed a chlorotic phenotype at a frequency of 33% (Shen *et al.*, 2007*b*). Interestingly, 32% of the ca also displayed chlorotic phenotypes (Fig. 1A). To test whether there was a correlation between the *ClpP4* expression level and chlorosis, the *ClpP4* transcript was analysed using RNA blot analysis with the result that in ca plants, the more severe the chlorotic phenotype, the lower the *ClpP4* transcript (Fig. 1B), while in CO plants, the more severe the chlorotic phenotype, the higher the *ClpP4* transcript (Fig. 1B). Western blot analyses generated similar results, indicating that the reduced expression of *ClpP4* and increased expression of *ClpP4* could both lead to the chlorotic phenotype in transgenic plants (Fig. 1C).

Overexpression of AtCHIP *lessened the chlorotic phenotypes of* ClpP4 *antisense and overexpression lines*

In the previous study, ClpP4 was found to be a substrate protein of the E3 ligase AtCHIP (Shen *et al.*, 2007*b*). To further investigate the roles of AtCHIP in ClpP4 function, *AtCHIP* was overexpressed in ca and CO plants. The expression and steady level of *AtCHIP* was analysed by RNA blot in ca and CO plants, and by western blot in caA and COA plants. As can be seen in Fig. 1D, E, all of the tested caA and

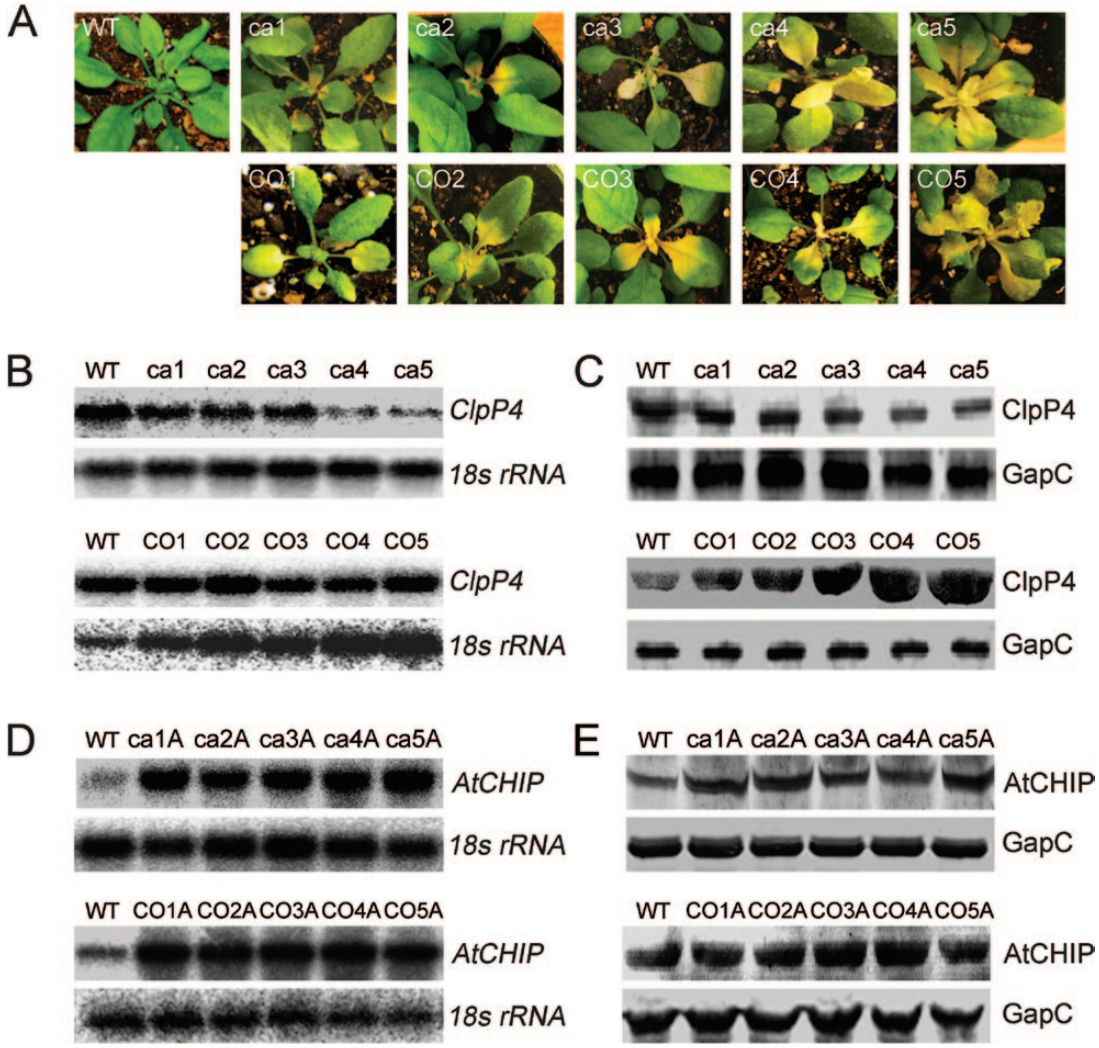

Fig. 1. Phenotypes and molecular characterisation of the transgenic plants. (A) Phenotypes of wild-type (WT), *ClpP4* antisense (ca1 to ca5), and *ClpP4*-overexpressing (CO1 to CO5) plants. (B) RNA blot analyses. The 18S rRNA was used as the RNA loading control. (C) Western blot analyses. The cytosolic glyceraldehyde-3-phosphate-dehydrogenase (GapC) was used as the protein loading control. (D) RNA blot analyses of *AtCHIP* overexpression. **E**. Western blot analyses of *AtCHIP* overexpression (this figure is available in colour at *JXB* online).

COA plants exhibited high levels of *AtCHIP* transcription and translation, indicating the successful overexpression of *AtCHIP* in ca and CO plants.

As shown in Fig. 2A, B, ca5A and CO5A plants displayed less severe chlorotic phenotypes than did their parental plants ca5 and CO5, indicating that the chlorotic phenotypes of ca and CO plants could be partially rescued by the overexpression of *AtCHIP*. To investigate whether this phenomenon was related to the steady level of ClpP4, the protein level of ClpP4 in caA and COA plants was examined. In caA plants, the steady levels of ClpP4 increased by more than 20% compared to those in ca plants, whereas in COA plants, the levels of ClpP4 decreased by ~50% compared to those of their parental plants (Fig. 2C–E), indicating a causal relationship between the levels of chlorosis and the changes in the steady levels of ClpP4 in caA and COA plants. The overexpression of *AtCHIP* in ca plants helped to increase the level of ClpP4 closer to that found in wild-type plants, whereas the overexpression of *AtCHIP* in COA plants helped to decrease the steady-state levels of ClpP4 to those found in wild-type plants, which explains why the overexpression of *AtCHIP* lessened the chlorotic phenotype in both types of transgenic plants.

AtCHIP interacts with ClpP3 and ClpP5 and is responsible for Clp subunit homeostasis

As previously demonstrated, the overexpression of *ClpP4* leads to decreased levels of other Clp subunits, i.e. ClpP3 and ClpP5, leading to reduced levels of functional Clp protease in chloroplasts and a chlorotic phenotype (Shen *et al.*, 2007*b*). Because ca plants displayed a similar phenotype, the impact of a reduced expression of *ClpP4* on the steady-state level of other Clp subunits was determined. Analysis of the steady-state levels of ClpP3, ClpP5, ClpP6 and ClpC1 indicated that, except for ClpC1, the other three Clp subunits increased in ca plants. The effects of *AtCHIP* overexpression on the steady-state levels of these Clp subunits in both cA and CO plants were the same, bringing the steady-state levels of other Clp subunits closer to those found in wild-type plants (Fig. 3A–C). The changes in the steady-state levels of ClpP3 and ClpP5 in *ClpP4*/*AtCHIP* co-overexpressing plants indicate

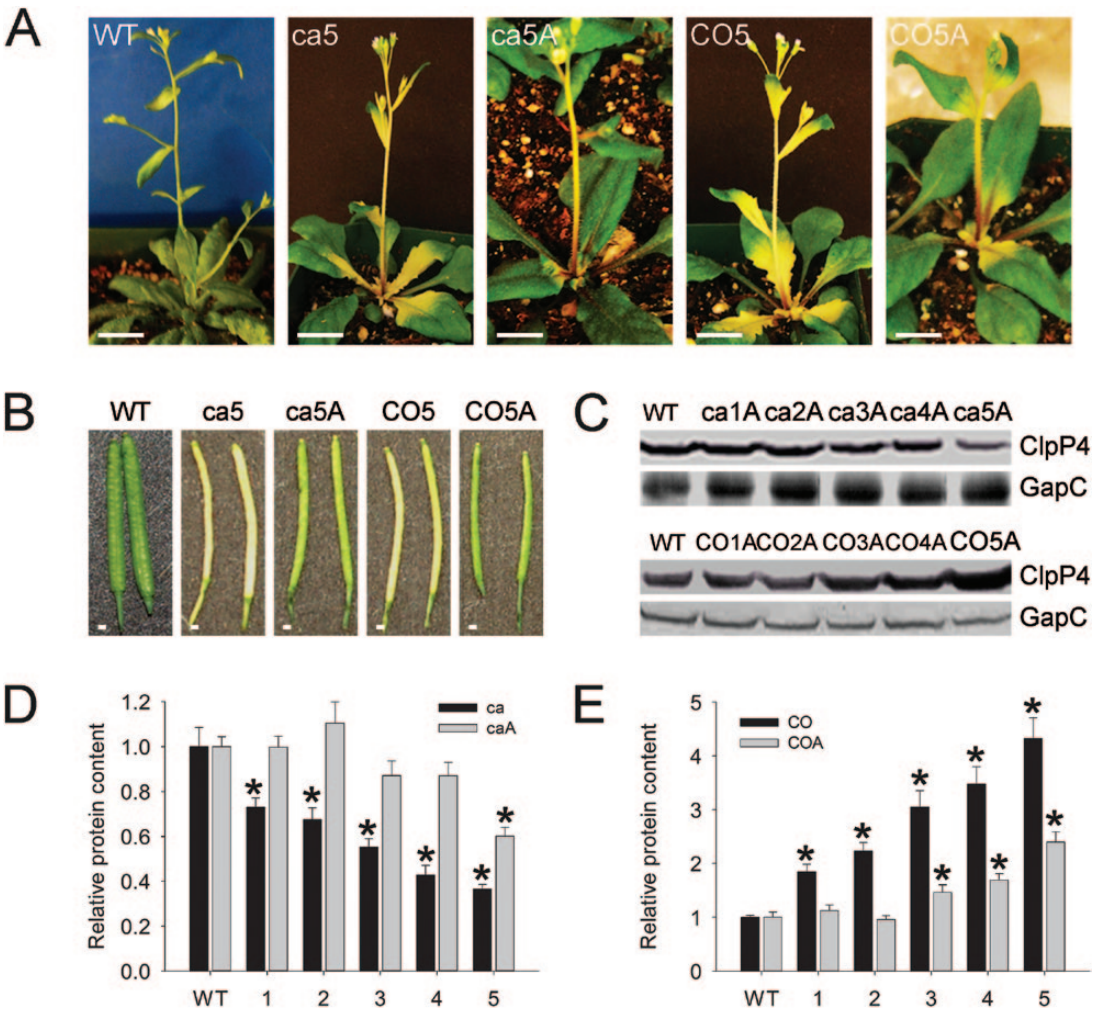

Fig. 2. The overexpression of *AtCHIP* in *ClpP4* antisense and in *ClpP4*-overexpressing plants lessens the chlorotic phenotypes. (A) Phenotypes of wild-type (WT), *ClpP4* antisense (ca5), *AtCHIP*-overexpressing/*ClpP4* antisense (ca5A), *ClpP4*-overexpressing (CO5), and *AtCHIP*/*ClpP4* co-overexpressing (CO5A) plants. (B) Phenotypes of seed pods. (C) Western blot analyses of the steady-state level of ClpP4. (D) Quantitative analyses of the steady-state levels of ClpP4. (E) Quantitative analyses of the steady-state levels of ClpP4 (this figure is available in colour at *JXB* online).

the possibility that, like ClpP4, both ClpP3 and ClpP5 could be substrates of AtCHIP. A yeast two-hybrid analysis was therefore conducted to test for interactions between AtCHIP and ClpP3 or ClpP5. AtCHIP interacted with ClpP3 and ClpP5 in yeast cells (Fig. 3D). *In vitro* ubiquitylation experiments were also conducted, and ClpP3 and ClpP5 were substrates of AtCHIP, similar to ClpP4 (Fig. 4). In the previous study, no change was seen in the steady-state level of ClpP6 in CO plants (Shen *et al.*, 2007*b*), but this time there was a reduced level of ClpP6 in CO plants (Fig. 3C). However, unlike what happened to ClpP3 and ClpP5, *AtCHIP* overexpression in CO plants did not increase the level of ClpP6, indicating a major difference in the response of Clp subunits to *AtCHIP* overexpression. In the case of ClpC1, a different response was observed. Both the reduced and increased expression of *ClpP4* decreased the steady-state level of ClpC1, and the overexpression of *AtCHIP* increased the steady-state level of ClpC1 in ca and CO plants (Fig. 3A–C). These results suggest that AtCHIP might play an important role in the homeostasis of some Clp subunits, probably by the ubiquitylation of precursors of ClpP3, ClpP4, and ClpP5 in the cytosol, thereby leading to their degradation in plant cells.

Chloroplast development and function were impaired in ClpP4 *antisense and* ClpP4*-overexpressing plants, and the overexpression of* AtCHIP *could reverse these traits*

Chloroplast development was previously found to be blocked in CO plants because no functional chloroplast could be found in the chlorotic regions of CO plants (Shen *et al.*, 2007*b*). To determine whether the chloroplast structure is also affected in *ClpP4* antisense plants, the chloroplast structure in ca plants was analysed and compared to the chloroplast structure of CO plants. No functional chloroplasts were found in the chlorotic tissues of ca5 or CO5 plants (Fig. 5A). However, when the rescued tissues (less chlorotic) of caA5 and COA5 plants were analysed, relatively normal chloroplasts were observed (Fig. 5A). The photosynthetic capacity was also examined in ca5, CO5, ca5A, CO5A, and wild-type plants. The photosynthetic rates, stomatal conductance, and transpiration rates significantly decreased in ca5 and CO5 plants, whereas in ca5A and CO5A plants, the photosynthetic rates, stomatal conductance, and transpiration rates increased but were still lower than the levels that were found in wild-type plants (Fig. 5 B, C). These data clearly demonstrate that the impaired function of chloroplasts in ca5 and CO5 plants could be partly restored by the overexpression of *AtCHIP* in ca and CO plants.

The steady-state levels of many chloroplast proteins were reduced in ClpP4 *antisense and overexpressing plants, and the overexpression of* AtCHIP *could increase the steady-state levels of these proteins*

To further study the effect of the AtCHIP–ClpP4 interaction on chloroplast function, the steady-state levels of a group of chloroplast proteins were analysed in ca5, ca5A, CO5, CO5A, and wild-type plants using western blotting. The

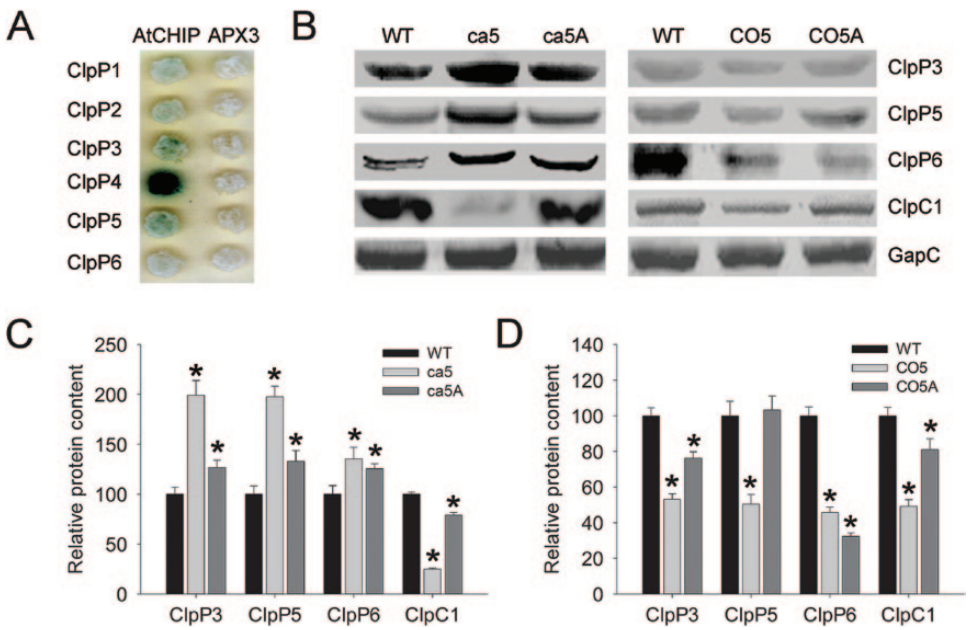

Fig. 3. *AtCHIP*-overexpression affects the steady-state levels of other Clp catalytic subunits and the protein–protein interactions between AtCHIP and ClpP3, ClpP4, or ClpP6. The results of one of three independent experiments are shown. (A) Protein–protein interactions between the AtCHIP and Clp catalytic subunits in the yeast two-hybrid system. (B) Western blot analyses of the steady-state levels of ClpP3, ClpP5, ClpP6, and ClpC1 in wild-type (WT), *ClpP4* antisense line 5 plants (ca5), *AtCHIP*-overexpressing/ClpP4 antisense plants (ca5A), *ClpP4*-overexpressing plants (CO5), and *AtCHIP/ClpP4* co-overexpressing plants (CO5A). (C) Quantitative analyses of the steady-state levels of ClpP3, ClpP5, ClpP6, and ClpC1 in *ClpP4* antisense plants and *AtCHIP*-overexpressing/*ClpP4* antisense plants. (D). Quantitative analyses of the steady-state levels of ClpP3, ClpP5, ClpP6, and ClpC1 in *ClpP4*-overexpressing plants and *AtCHIP/ClpP4* co-overexpressing plants. Asterisks indicate significant differences as compared with the wild-type (*$P < 0.05$) (this figure is available in colour at *JXB* online).

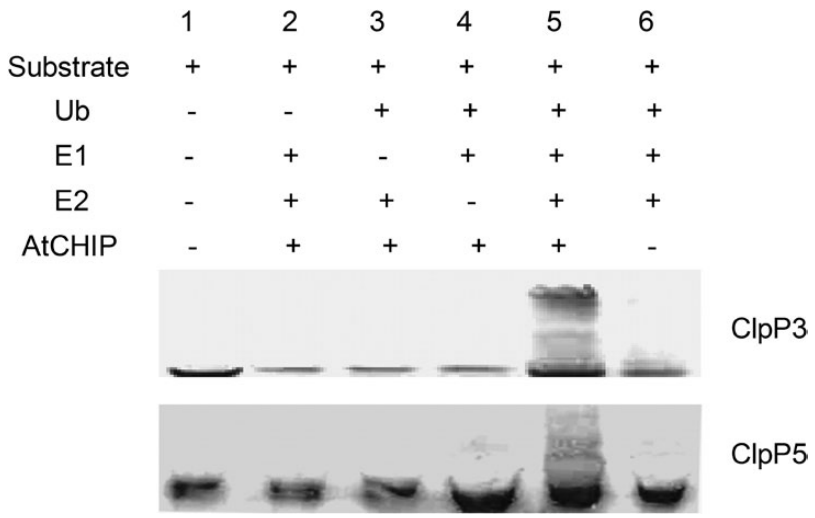

Fig. 4. AtCHIP can ubiquitylate ClpP3 and ClpP5 *in vitro*. The reaction mixture includes AtCHIP as the E3 ligase, AtUBC8 as an E2 conjugase, rabbit E1 as an activase, and bovine ubiquitin, ClpP3, or ClpP5 as a substrate. Antibodies against ClpP3 and ClpP5 were used in the western blot. Ub, ubiquitin; E1, ubiquitin-activating enzyme; E2, ubiquitin conjugase. Lanes 1–6 represent six different ubiquitylation reaction conditions.

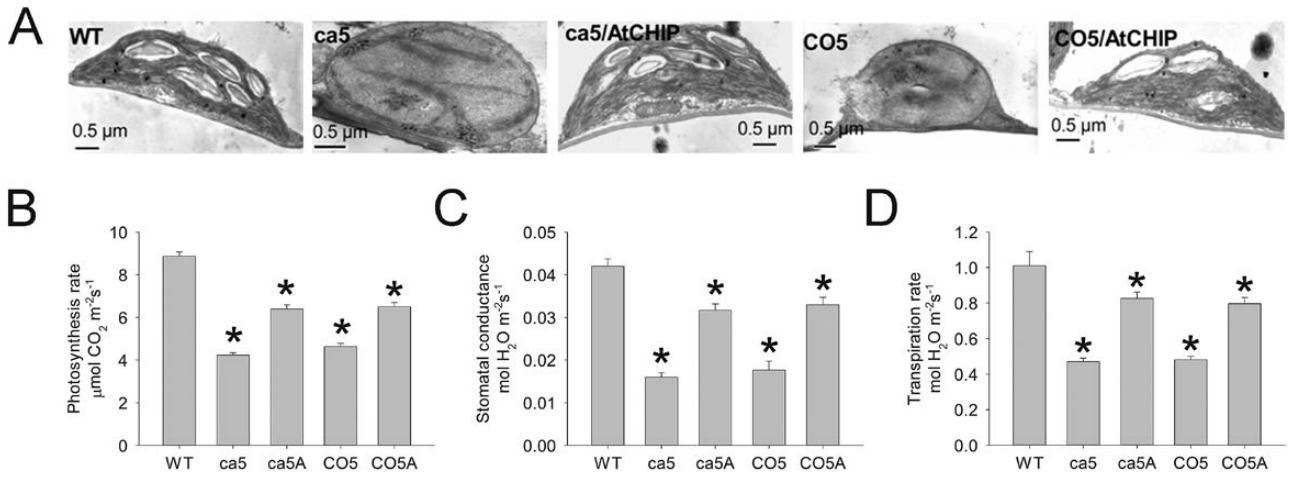

Fig. 5. (A) Chloroplast structures and (B-D) photosynthetic performance of wild-type (WT), *ClpP4* antisense (ca5), *AtCHIP* overexpressing/*ClpP4* antisense (ca5A), *ClpP4*-overexpressing (CO5), and *AtCHIP/ClpP4* co-overexpressing plants (CO5A). The measurements were taken when plants were 28 days old. For photosynthetic determination, five replicates were taken for each sample. Asterisks indicate significant differences as compared with the wild-type plant (*P <0.05).

steady-state levels of PSI and PSII proteins decreased in ca5 and CO5 plants to some extent but increased in ca5A and CO5A plants (Fig. 6), suggesting that the reduced level of PSI and PSII proteins in ca or CO plants could be reversed by the overexpression of *AtCHIP*. In addition to the selected PSI and PSII proteins, other chloroplast proteins displayed varied responses to changes in *ClpP4* and *AtCHIP* overexpression. For instance, the responses of the Rieske Fe-S protein PetC and the Rubisco large subunit protein RbcL were the same as those of PSI and PSII proteins (Fig. 6). The GTPase TypA/BiPA and the Zn-metallo proteases FtsH1 and FtsH2, however, seemed to respond to changes in the steady-state levels of ClpP4 but not to the overexpression of *AtCHIP* because the steady-state levels of these proteins increased in ca5 and CO5 plants compared to those in wild-type plants but did not change much in ca5A and CO5A plants in which *AtCHIP* was overexpressed. The level of the ATP-independent serine-type protease Deg1 did not show a significant change between transgenic plants and wild-type plants (Fig. 6).

Changes in ClpP4 also affect chloroplast development in tobacco plants, and the chlorotic phenotypes could be reversed by the overexpression of AtCHIP *in tobacco plants*

Based on data that were obtained from transgenic *Arabidopsis* plants, it was inferred that AtCHIP might play an important role in the homeostasis of Clp proteins and therefore could partially restore the impaired chloroplast development and function that are caused by ClpP4 deficiency or ClpP4 overproduction. To test this hypothesis, similar experiments were conducted with tobacco plants. More than 50% of the Tca and TCO plants displayed a chlorotic phenotype (Fig. 7A). The *Arabidopsis CHIP* gene was then introduced into these chlorotic Tca and TCO plants. Transcript and protein levels of

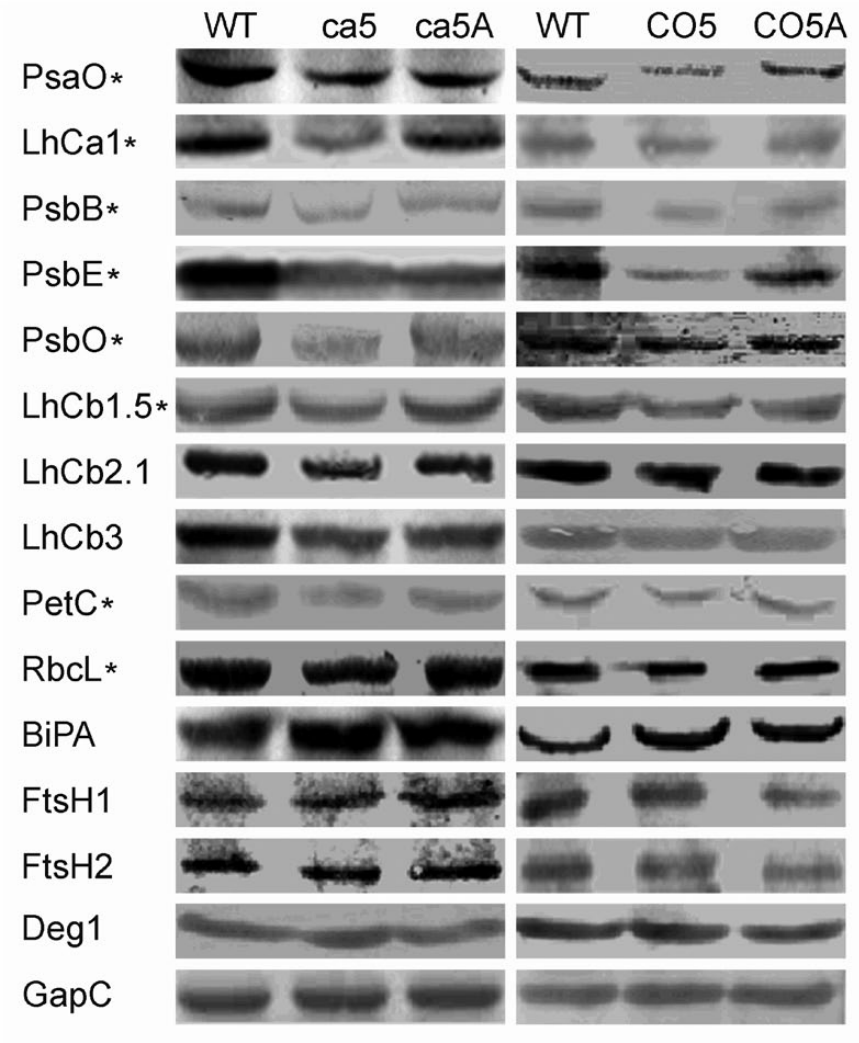

Fig. 6. Analysis of the steady-state levels of some chloroplast proteins in *ClpP4* antisense (ca5), *AtCHIP*-overexpressing/*ClpP4* antisense (ca5A), *ClpP4*-overexpressing (CO5), and *AtCHIP/ClpP4* co-overexpressing (CO5A) plants. Total leaf proteins were extracted from transgenic and wild-type plants. Proteins were separated by SDS-PAGE and transferred to western blots. Specific antibodies against PsaO, LhCa1, PsbB, PsbE, PsbO, LhCb1.5, LhCb2.1, LhCb3, PetC, RbcL, BiPA, Ftsh1, Ftsh2, and Deg1 were used in blots, and GapC was used as protein loading controls. Asterisks indicate proteins that were restored by ATCHIP expression.

ClpP4 and AtCHIP were measured in Tca, TCO, TcaA, and TCOA plants by RNA blot and western blot analyses, respectively. The *ClpP4* transcript decreased in Tca and TcaA plants but increased in TCO and TCOA plants compared to that in wild-type plants (Fig. 7C). The expression of the *AtCHIP* transcript increased in TcaA and TCOA plants (Fig. 7C). As expected, the steady-state level of ClpP4 decreased in Tca plants and increased in TCO plants, and similar to the results that were observed in *Arabidopsis*, the steady-state level of ClpP4 increased in TcaA plants and decreased in TCOA plants (Fig. 7D). To investigate whether AtCHIP also plays a role in the homeostasis of other Clp proteins in tobacco, steady-state levels of ClpP3, ClpP5, and ClpP6 in these transgenic tobacco plants were also analysed. The levels of ClpP3 and ClpP5 increased in Tca plants but decreased in TCO plants, while those in TcaA and TCOA plants returned to the wild-type level (Fig. 7E). The steady-state level of ClpP6, on the other hand, only responded to changes in ClpP4 but not AtCHIP, which is similar to that found in *Arabidopsis*. These data suggest that the regulatory role of AtCHIP in the homeostasis of Clp proteins also applies to the tobacco system. In addition, the ability of *AtCHIP* overexpression to rescue the chlorotic phenotype of Tca and TCO plants (Fig. 7A) could also be demonstrated by measuring the photosynthetic performance of TcaA and TCOA plants (Fig. 8). It is clear that *AtCHIP* overexpression could increase the photosynthetic performance of Tca and TCO tobacco plants (Fig. 8).

Discussion

Proteases have been shown to play essential roles in chloroplast internal protein quality control. They are either synthesized within the chloroplasts or within the endoplasmic reticulum in cytosol, and then transported to chloroplasts (Vierstra, 2009; Jarvis and López-Juez, 2014). The ClpP subunits ClpP3, ClpP4, and ClpP5 are nuclear-encoded chloroplast proteins (Peltier *et al.*, 2004); these chloroplast proteases are maintained by a combination of proper biogenesis

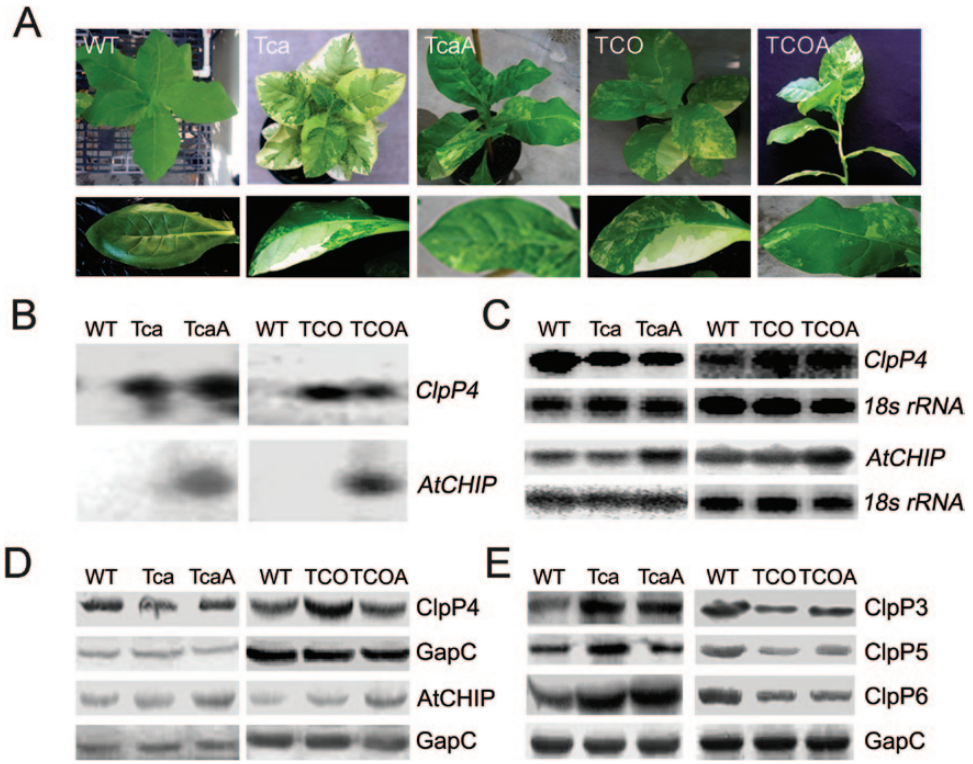

Fig. 7. Phenotype and characterisation of tobacco plants with reduced or increased expression of *ClpP4*. (A) Phenotypes of wild-type (WT), *ClpP4* antisense line (Tca), *AtCHIP* overexpressing/*ClpP4* antisense line (TcaA), *ClpP4*-overexpressing line (TCO), and *AtCHIP/ClpP4* co-overexpressing tobacco plants (TCOA). (B) DNA blot analyses. (C) RNA blot analyses. (D and E) Western blot analyses. (F and G) Quantitative analyses of the changes of Clp proteolytic subunits (this figure is available in colour at *JXB* online).

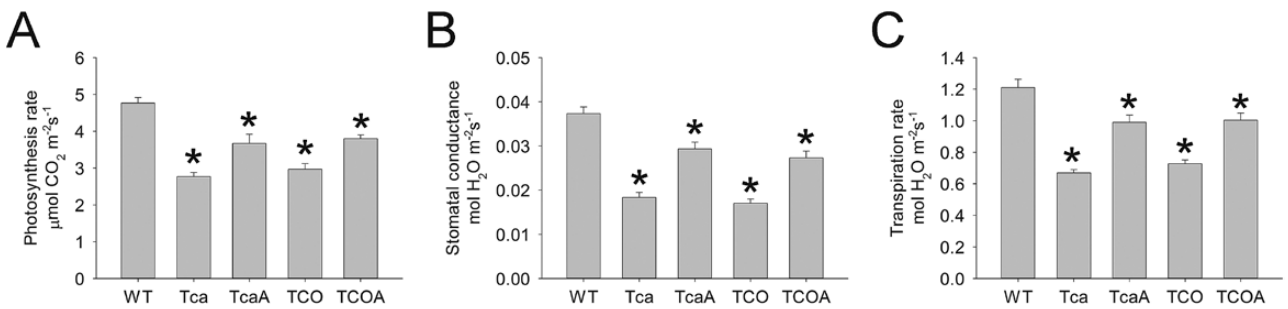

Fig. 8. Photosynthetic performance of wild-type (WT), *ClpP4* antisense line (Tca), *AtCHIP* overexpressing/*ClpP4* antisense (TcaA), *ClpP4*-overexpressing (TCO), and *AtCHIP/ClpP4* co-overexpressing (TCOA) tobacco plants. (A) Photosynthetic rate. (B) Stomatal conductance. (C) Transpiration. The measurements were taken when plants were 28 days old. For photosynthetic determination, five replicates were taken for each sample. Asterisks indicate significant differences as compared with the wild-type (*$P < 0.05$).

processes in the cytosol and homeostasis in the chloroplasts that together guarantee the functional complex in the chloroplasts. The ubiquitin-26S proteasome system in particular displays exceedingly good protein degradation and turnover machinery in the cytosol (Vierstra, 2009). In this study, the process by which cytosole-located E3 ligase AtCHIP regulates Clp proteolytic subunit homeostasis in plants was investigated. Both the overexpression of *ClpP4* and the antisense repression of *ClpP4* led to the same phenotype of chlorotic leaves and stems in transgenic plants (Figs 1A and 7A). Surprisingly, overexpression of *AtCHIP* could also lessen the severity of chlorosis as demonstrated in the ca and CO plants (Figs. 2 and 7). More importantly, the steady-state levels of ClpP4 in both can and CO plants could be returned to a level that is closer to that found in wild-type plants (Fig. 2), which explains why the overexpression of *AtCHIP* could lessen the severity of chlorosis of both ca and CO plants. However, it is difficult to explain at the molecular level why overexpressing *AtCHIP* would increase the steady-state levels of ClpP4 in ca plants or decrease the steady-state levels of ClpP4 in CO plants, consequently lessening the chlorotic phenotype of ca and CO plants.

Each subunit of the Clp protease complex has a unique contribution to Clp core functions (Halperin *et al.*, 2001; Andersson *et al.*, 2009; Kim *et al.*, 2013), and a change in the production of one subunit might disrupt the overall structure of the Clp protease complex (Shen *et al.*, 2007*b*). For example, the antisense repression of *ClpP6* led to a reduced steady-state level of ClpP3 and increased steady-state levels of ClpP4 and ClpP5 (Sjogren *et al.*, 2006; Kim *et al.*, 2013),

and the loss of ClpP3 led to the overaccumulation of ClpP1, ClpP5, and ClpP6 (Kim *et al.*, 2013). To determine whether one Clp protein affects the level of one or more of the others, the steady-state levels of ClpP3, ClpP5, and ClpP6 were determined in *ClpP4* transgenic plants. Indeed, the steady-state levels of ClpP3, ClpP5, and ClpP6 increased in ca plants, while *AtCHIP* overexpression in these plants returned the level of ClpP4 closer to that found in wild-type plants (Fig. 3B). Interestingly, in CO plants, the steady-state levels of ClpP3, ClpP5, and ClpP6 decreased, whereas when *AtCHIP* was overexpressed in these plants, the steady-state levels of ClpP3 and ClpP5 but not ClpP6 returned to those found in wild-type plants (Fig. 3C). These data indicate that there are differences in the responses of Clp catalytic subunits to *AtCHIP* overexpression, with ClpP3 and ClpP5 appearing to be more similar to each other than to the ClpP6 subunit. In both ca and CO plants, the overexpression of *AtCHIP* increased the steady-state level of the chaperone subunit of the Clp complex, ClpC1 (Figs. 3B, C), indicating that this regulatory subunit of the Clp complex is positively affected by the overexpression of *AtCHIP* in both ca and CO plants.

The chlorotic phenotype that was displayed by both ca and CO plants is due to a defect in chloroplast development, because no functional chloroplasts could be found in the chlorotic regions of either ca or CO plants (Fig. 5A). The photosynthetic performance of both ca and CO plants was also severely compromised (Fig. 5B–D). Overexpression of *AtCHIP* in these plants could enable these plants to re-establish some functional chloroplasts and increase the photosynthetic performance of both ca and CO plants (Fig. 5). The loss of functional chloroplasts and decreased photosynthetic performance in these plants are also likely due to the disrupted chloroplast biogenesis by many other reduced chloroplast proteins (Fig. 6). Again, the overexpression of *AtCHIP* can partially recover these proteins, indicating that AtCHIP may be involved in chloroplast protein regulation via an additional unknown mechanism. Interestingly, a similar regulatory mechanism was obtained in another model plant of tobacco system, suggesting the role of maintaining the subunits of Clp protease by AtCHIP are conserved (Figs. 7 and 8).

Typically, most nucleus-encoded chloroplast proteins are synthesized and translated in precursor form in cytosol before they are imported into chloroplasts. Previous research on the chloroplast target proteins of proteome systems has revealed AtCHIP regulation of the chloroplast protease subunit through degradation of the precursors in the cytosol (Shen *et al.*, 2007*b*; Yee and Goring, 2009). In the present paper, a model is produced to give a description of the regulatory mechanism. Overexpression of ClpP4 in plant cells may disrupt the efficient assembly of the Clp core complex in chloroplasts, triggering a response that may involve the down-regulation of other Clp subunits, such as ClpP3, ClpP5, and ClpP6, leading to the chlorotic phenotype. However, if *AtCHIP* is overexpressed in CO plants, the extra amount of ClpP4 accumulated in plant cells may be removed, permitting the more balanced ratios of other Clp subunits to form a functional Clp core complex, and leading to the formation of more Clp core complexes. In ca plants, the reduction of ClpP4 may trigger a compensation mechanism, increasing the steady-state levels of ClpP3, ClpP5, and ClpP6. Because the P-ring in the Clp core complex exists at a ratio of 1:2:3:1 (ClpP3 to ClP4 to ClpP5 to ClpP6), accumulated ClpP3, ClpP5, and ClpP6 compete to enter the P-ring, leaving the possibility of functional P-ring formation less likely;

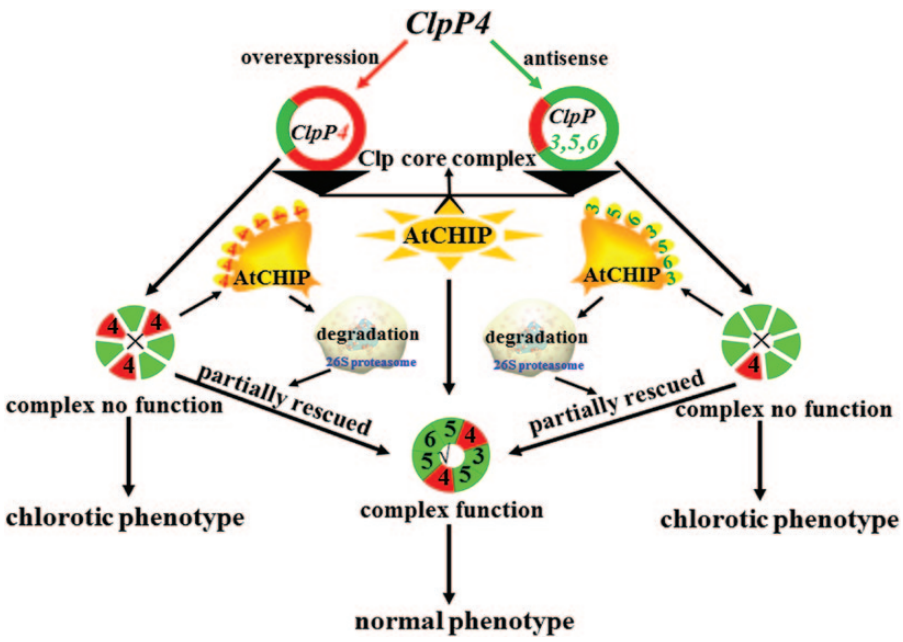

Fig. 9. A proposed model for the regulation of AtCHIP in Clp proteolytic subunits homeostasis. When ClpP4 is overexpressed in plants, AtCHIP targets the abundant precursors of ClpP4 in the cytosol for degradation, thereby maintaining the balance of Clp core subunits in the chloroplast and partially rescuing the chlorotic phenotype of *ClpP4*-overexpressing plants. In *ClpP4* antisense plants, in which ClpP3, ClpP5, and ClpP6 are overaccumulated, AtCHIP then targets the abundant precursors of ClpP3 and ClpP5 for degradation, thereby maintaining the homeostasis of Clp core subunits in the chloroplast and partially rescuing the chlorotic phenotype of ClpP4 antisense plants (this figure is available in colour at *JXB* online).

thus, plants are chlorotic. However, *AtCHIP* overexpression may help remove the extra Clp proteolytic subunits, increasing the chance of the remaining ClpP4 entering the P-ring and forming a functional Clp core complex. The ratio of Clp core subunits in the functional P-ring in chloroplast should not be impacted in restoration of Clp activity in *AtCHIP* overexpression lines according to previous studies (Olinares *et al*., 2011a). Thus, overall, these data suggest that the E3 ligase AtCHIP can remove accumulated Clp proteolytic subunits in plant cells, consequently maintaining the homeostasis of Clp protease in chloroplasts (Fig. 9).

Supplementary data

Supplementary data can be found at *JXB* online.

Supplementary Table S1. DNA sequences of primers used in the yeast two-hybrid assay.

Acknowledgements

This project was supported by grants from the National Natural Science Foundation of China (31170793, 31300259, 31402140) and Zhejiang Province LY15C020002; the State Key Laboratory Breeding Base for Zhejiang Sustainable Pest and Disease Control (2010DS700124-KF1405); the International Cooperation, Innovation and Upgrading programs of Zhejiang Academy of Agricultural Sciences (2014CX005); and the Shaoxing 330 Overseas Elites Program.

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