The E3 ligase AtCHIP ubiquitylates FtsH1, a component of the chloroplast FtsH protease, and affects protein degradation in chloroplasts

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Summary

The Arabidopsis E3 ligase AtCHIP was found to interact with FtsH1, a subunit of the chloroplast FtsH protease complex. FtsH1 can be ubiquitylated by AtCHIP *in vitro*, and the steady-state level of FtsH1 is reduced in *AtCHIP*-over-expressing plants under high-intensity light conditions, suggesting that the ubiquitylation of FtsH1 by AtCHIP might lead to the degradation of FtsH1 *in vivo*. Furthermore, the steady-state level of another subunit of the chloroplast FtsH protease complex, FtsH2, is also reduced in *AtCHIP*-over-expressing plants under high-intensity light conditions, and FtsH2 interacts physically with AtCHIP *in vivo*, suggesting the possibility that FtsH2 is also a substrate protein for AtCHIP in plant cells. A substrate of FtsH protease *in vivo*, the photosystem II reaction center protein D1, is not efficiently removed by FtsH in *AtCHIP*-over-expressing plants under high-intensity light conditions, supporting the assumption that FtsH subunits are substrates of AtCHIP *in vivo*, and that *AtCHIP* over-expression may lead to a reduced level of FtsH in chloroplasts. AtCHIP interacts with cytosolic Hsp70 and the precursors of FtsH1 and FtsH2 in the cytoplasm, and Hsp70 also interacts with FtsH1, and these protein–protein interactions appear to be increased under high-intensity light conditions, suggesting that Hsp70 might be partly responsible for the increased degradation of the substrates of Hsp70, such as FtsH1 and FtsH2, in *AtCHIP*-over-expressing plants under high-intensity light conditions. Therefore, AtCHIP, together with Hsp70, may play an important role in protein quality control in chloroplasts.

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

Introduction

Protein degradation is a key component of protein homeostasis in cellular metabolism. If a protein is not folded correctly or is damaged under stress conditions, it is usually caught by molecular chaperones such as Hsp70 and Hsp90, which help refold or repair the aberrant protein (Wickner *et al.*, 1999). However, if the refolding or repair effort fails, the protein is then degraded (Wickner *et al.*, 1999). Several chaperone co-factors play important roles in determining the outcome of the chaperone's action, and are therefore critical regulators of protein quality control in eukaryotic cells (Gottesman *et al.*, 1997; Höhfeld *et al.*, 2001). One such chaperone co-factor in animal cells is called CHIP, and regulates the activities of both Hsp70 and Hsp90 (Murata *et al.*,

2001). When CHIP binds to Hsp70 or Hsp90, it inhibits their chaperone activities. Furthermore, CHIP proteins are chaperone-dependent ubiquitin E3 ligases that induce ubiquitylation of the substrate proteins of Hsp70 and Hsp90 and stimulate their degradation through the 26S proteasome (Connell *et al.*, 2001; Demand *et al.*, 2001; Meacham *et al.*, 2001). Consequently, CHIP functions as a degradation factor in protein turnover metabolism, and plays a key role in cellular protein homeostasis in animal cells (Cyr *et al.*, 2002; Höhfeld *et al.*, 2001).

CHIP proteins are conserved throughout evolution, and CHIP homologs have been identified in various animals and plants (Patterson, 2002). We previously discovered

that the Arabidopsis CHIP-like protein, AtCHIP, plays important roles in the plant response to abiotic stress conditions (Yan *et al.*, 2003). Over-expression of *AtCHIP* in transgenic plants leads to increased sensitivity to both high- and low-temperature conditions (Yan *et al.*, 2003). To understand the molecular mechanism of the involvement of AtCHIP in the stress response, we identified proteins that physically interact with AtCHIP using the yeast two-hybrid technique. One AtCHIP-interacting protein is an A subunit of protein phosphatase 2A (i.e. PP2AA3). We further discovered that AtCHIP can add one ubiquitin molecule to A subunits of PP2A *in vitro* (Luo *et al.*, 2006), and that the activity of PP2A is increased in *AtCHIP*-over-expressing plants, suggesting that the ubiquitylation of A subunits by AtCHIP may activate PP2A. As PP2A has been shown to be involved in the low-temperature response in plants (Monroy *et al.*, 1998), there might be a connection between the cold sensitivity of *AtCHIP*-over-expressing plants and the increased PP2A activity in these plants (Luo *et al.*, 2006).

Another AtCHIP-interacting protein, ClpP4, is a proteolytic subunit of chloroplast Clp protease (Shen *et al.*, 2007). The Clp protease is an ATP-dependent serine protease that is involved in degrading proteins in chloroplast stroma (Adam and Clarke, 2002; Adam *et al.*, 2006; Sakamoto, 2006; Sjogren *et al.*, 2006). We discovered that AtCHIP can ubiquitylate ClpP4 *in vitro*, and the steady-state level of ClpP4 is reduced in *AtCHIP*-over-expressing plants under high-intensity light conditions (Shen *et al.*, 2007). In contrast to the A subunits of PP2A, where ubiquitylation by AtCHIP leads to activation of PP2A (Luo *et al.*, 2006), ClpP4 ubiquitylation by AtCHIP leads to degradation of ClpP4 under high-intensity light conditions (Shen *et al.*, 2007), suggesting that AtCHIP may be involved in chloroplast protein quality control through ubiquitylation and degradation of chloroplast-targeted protease precursors. The validity of this suggestion is strengthened by the results of this study on the third AtCHIP-interacting protein, FtsH1. FtsH1, together with FtsH2, FtsH5 and FtsH8, comprise the thylakoid membrane-bound ATP-dependent FtsH complex (Adam *et al.*, 2006; Sakamoto, 2006; Zaltsman *et al.*, 2005), which is thought to degrade misfolded or damaged membrane proteins in chloroplast thylakoid membranes (Bailey *et al.*, 2002; Lindahl *et al.*, 2000; Sakamoto *et al.*, 2003). Like ClpP4, FtsH1 can be ubiquitylated by AtCHIP *in vitro*, and the steady-state level of FtsH1 is reduced in *AtCHIP*-over-expressing plants under high-intensity light conditions. Moreover, another FtsH subunit, FtsH2, is also reduced in *AtCHIP*-over-expressing plants under these conditions. In consequence, the removal of oxidatively damaged D1 protein of photosystem II (PSII), a substrate protein of FtsH protease *in vivo*, is impaired in these plants. These data highlight the importance of AtCHIP as a regulator of protein quality control in both cytoplasm and chloroplast.

Results

AtCHIP interacts with and ubiquitylates FtsH1

In order to study how AtCHIP is involved in the stress response in plants, we identified the proteins that interact with AtCHIP using the yeast two-hybrid technique. Among the 30 proteins identified, many are chloroplast proteins such as small subunit of Rubisco and light-harvesting chlorophyll *a/b* binding protein, in addition to ubiquitylation-related proteins such as ubiquitin molecules and E2 ubiquitin conjugases (Luo *et al.*, 2006). Two AtCHIP-interacting proteins, ClpP4 and FtsH1, might play important roles in protein quality control in chloroplasts, because they are proteolytic subunits of proteases in the chloroplast (Luo *et al.*, 2006). To test whether FtsH1 is a substrate protein for AtCHIP, we conducted an *in vitro* ubiquitylation experiment using AtCHIP as the E3 ligase and FtsH1 as the substrate. Because the full-length FtsH1 contains two transmembrane domains at its N-terminal side, which made its purification from a bacterial expression system difficult, we used a partial FtsH1 fragment that does not contain the two transmembrane domains as the substrate in the ubiquitylation reaction. As expected, the partial FtsH1 fragment was efficiently ubiquitylated by AtCHIP (Figure 1a). It appears that at least four or five ubiquitin molecules are added to the FtsH1 fragment *in vitro* (Figure 1a).

If FtsH1 is a substrate of AtCHIP *in vivo*, these two proteins should interact with each other. To test this possibility, we conducted co-precipitation experiments with protein extracts from wild-type and *AtCHIP*-over-expressing plants. The cellular extracts were first separated into two fractions by centrifugation. The supernatant fraction contains mainly soluble proteins including AtCHIP, whereas the pellet fraction contains organelles and membrane proteins including the thylakoid membrane-bound FtsH1. We used the supernatant fraction for the co-precipitation experiment. As expected, AtCHIP antibodies could pull down FtsH1 from the supernatant fraction (Figure 1b), but no FtsH1 was pulled down if no AtCHIP antibodies or unrelated antibodies (e.g. APX3 antibodies) were used in the co-precipitation experiments (Figure 1b). Interestingly, the FtsH1 pulled down by AtCHIP antibodies migrated with a molecular weight of about 76 kDa, which is the size of the full-length FtsH1 molecule (i.e. precursor), and the FtsH1 protein prepared from the membrane protein fraction migrated with a molecular weight of about 72 kDa, which is the size of the mature FtsH1 protein in thylakoid membranes. The difference is due to the removal of the transit peptide (approximately 4 kDa) from the FtsH1 precursor during import of FtsH1 into the chloroplast. Our data indicate that AtCHIP interacts with the precursor of FtsH1 in the cytoplasm. Furthermore, consistently more FtsH1 was pulled down by AtCHIP antibodies after a high-intensity light treatment of
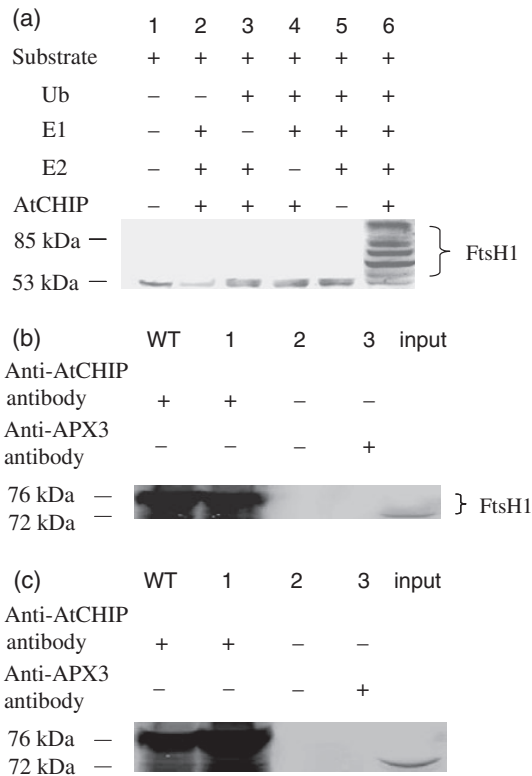
Figure 1. *In vitro* ubiquitylation of FtsH1 and co-precipitation of FtsH1 with AtCHIP.
(a) *In vitro* ubiquitylation of FtsH1. Ub, ubiquitin; E1, ubiquitin-activating enzyme; E2, ubiquitin conjugase. The polyubiquitylated FtsH1 proteins were recognized by the FtsH1 antibodies. Lanes 1–6 represent six different ubiquitylation reaction conditions.
(b) Co-precipitation of FtsH1 and AtCHIP with protein extracts from normal light-treated plants.
(c) Co-precipitation of FtsH1 and AtCHIP with protein extracts from high-intensity light-treated plants. WT, wild-type; lanes 1–3, protein extracts from the *AtCHIP*-over-expressing plant (O3486). The input lane was loaded with 50 µg of leaf membrane proteins. AtCHIP antibodies were used to pull down FtsH1 precursor from the supernatant fraction of protein extracts, and FtsH1 antibodies, which recognize both mature FtsH1 (72 kDa) and FtsH1 precursor (76 kDa), were used in the Western blot.

Figure 2. Steady-state levels of FtsH1 in wild-type and *AtCHIP*-over-expressing plants under normal and stress conditions.
(a) Normal conditions.
(b) Heat-stress conditions.
(c) High-intensity light conditions.
(d) Relative levels of FtsH1 in wild-type and five *AtCHIP*-over-expressing plants after high-intensity light treatment (obtained by analyzing the levels of FtsH1 normalized to the levels of APX3 shown in (c) using densitometry analysis). WT, wild-type; lanes 1–5, five independent *AtCHIP*-over-expressing plants, i.e. O3261, O3486, O264, O3122 and O476, respectively; APX3, ascorbate peroxidase 3 (a peroxisomal membrane-bound protein that serves as the loading control for Western blot).

level of FtsH1 in plant cells. To test this possibility, we conducted Western blot experiments with protein extracts from wild-type and *AtCHIP*-over-expressing plants. We found that, under normal growth and heat-stress conditions, the steady-state level of FtsH1 does not appear to be affected in *AtCHIP*-over-expressing plants (Figure 2a,b). However, after high-intensity light treatment, the steady-state level of FtsH1 is significantly reduced (Figure 2c), similar to what was observed for the steady-state level of ClpP4, which is also reduced under high-intensity light conditions in *AtCHIP*-over-expressing plants (Shen *et al.*, 2007). These data support our hypothesis that FtsH1 is a substrate of AtCHIP and that ubiquitylation of FtsH1 by AtCHIP might lead to its degradation *in vivo* under high-intensity light conditions.

The transcript level of FtsH1 *is not reduced in* AtCHIP-*over-expressing plants*

To rule out the possibility that the reduced level of FtsH1 in *AtCHIP*-over-expressing plants under high-intensity light conditions is due to reduced expression of *FtsH1* at the transcription level, we conducted Northern blot experiments to analyze the *FtsH1* transcript after high-intensity light treatment. Our data indicate that there are no major differences at the transcript level between wild-type and

the *AtCHIP*-over-expressing plants (compare lane 1 to lane WT in Figure 1c), whereas under normal light conditions, we always pulled down a roughly equal amount of FtsH1 from an equal amount of total proteins in the supernatant fraction (Figure 1b). Therefore, *AtCHIP* over-expression appears to increase AtCHIP–FtsH1 interaction under high-intensity light conditions.

The steady-state level of FtsH1 *is reduced in* AtCHIP-*over-expressing plants*

As the partial FtsH1 fragment can be ubiquitylated by AtCHIP *in vitro* and FtsH1 can interact with AtCHIP *in vivo*, FtsH1 might be a substrate protein for AtCHIP *in vivo*. If so, over-expression of *AtCHIP* might affect the steady-state
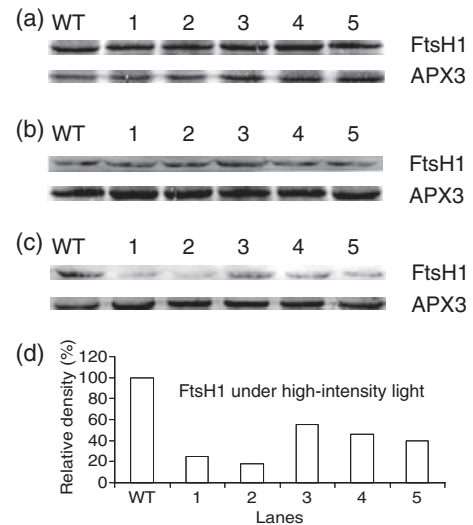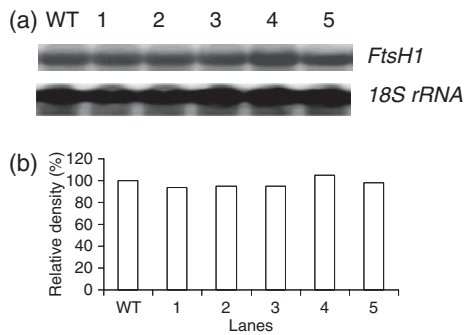
(a) WT 1 2 3 4 5

FtsH1

18S rRNA

(b)

Figure 3. Steady-state levels of *FtsH1* transcript in wild-type and *AtCHIP*-over-expressing plants after high-intensity light treatment.
(a) Steady-state levels of *FtsH1* transcript in wild-type and *AtCHIP*-over-expressing plants after high-intensity light treatment.
(b) Relative levels of *FtsH1* transcript in wild-type and five *AtCHIP*-over-expressing plants after high-intensity light treatment (obtained by analyzing the levels of *FtsH1* transcript normalized to the levels of 18S rRNA shown in (a) using densitometry analysis). WT, wild-type; lanes 1–5, five independent *AtCHIP*-over-expressing plants; 18S rRNA, RNA loading control.

AtCHIP-over-expressing plants (Figure 3), suggesting that the difference observed at the protein level between wild-type and *AtCHIP*-over-expressing plants is probably due to control at the post-transcriptional level.

The steady-state level of FtsH2 is also reduced in AtCHIP-*over-expressing plants*

In the chloroplast, FtsH1, FtsH2, FtsH5 and FtsH8 form hetero-oligomeric hexamers that function as an ATP-dependent metalloprotease on the thylakoid membrane (Adam *et al.*, 2006; Sakamoto, 2006). Because these four subunits share high sequence similarity, we thought that they might also be substrate proteins of AtCHIP. To test this possibility, we analyzed the steady-state level of the most abundant FtsH subunit, FtsH2, in *AtCHIP*-over-expressing plants. Under normal growth conditions, the steady-state level of FtsH2 was not different between wild-type and *AtCHIP*-over-expressing plants (Figure 4a); however, after high-intensity light treatment, the steady-state level of FtsH2 decreased in *AtCHIP*-over-expressing plants (Figure 4b), and the reduction varied from 35% to about 60% (Figure 4c). Although this decrease may be due to other reasons, AtCHIP-mediated protein degradation is a probable cause considering the fact that AtCHIP also interacts with FtsH2 physically *in vivo* as demonstrated by the pull-down experiment shown in Figure 4(d). Furthermore, AtCHIP also interacts with the precursor of FtsH2 in the cytoplasm, because the FtsH2 protein pulled down by AtCHIP antibodies has a molecular weight of about 74 kDa, whereas the FtsH2 extracted directly from thylakoid membranes has a molecular weight of about 70 kDa (Figure 4e), and this difference is probably due to the transit peptide that is removed from FtsH2 precursor
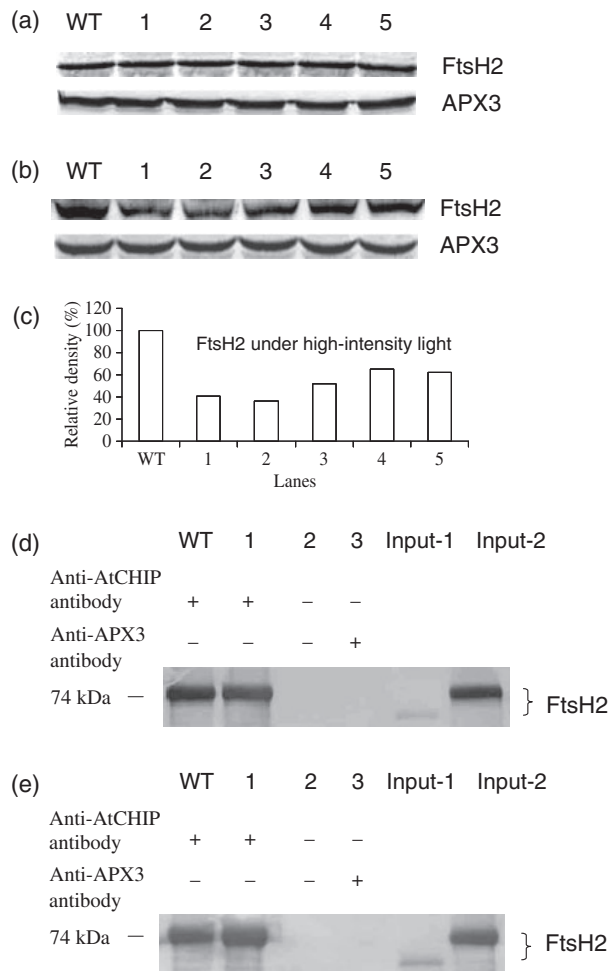
(a) WT 1 2 3 4 5

FtsH2

APX3

(b) WT 1 2 3 4 5

FtsH2

APX3

(c)

(d)

(e)

Figure 4. Steady-state levels of FtsH2 in wild-type and *AtCHIP*-over-expressing plants and co-precipitation of FtsH2 with AtCHIP.
(a) Steady-state levels of FtsH2 in wild-type and *AtCHIP*-over-expressing plants under normal growth conditions.
(b) Steady-state levels of FtsH2 in wild-type and *AtCHIP*-over-expressing plants after high-intensity light treatment.
(c) Relative levels of FtsH2 in wild-type and five *AtCHIP*-over-expressing plants after high-intensity light treatment (obtained by analyzing the levels of FtsH2 normalized to the levels of APX3 shown in (b) using densitometry analysis). WT, wild-type; lanes 1–5, five independent *AtCHIP*-over-expressing plants; APX3, ascorbate peroxidase 3.
(d) Co-precipitation of FtsH2 and AtCHIP with protein extracts from plants under normal conditions.
(e) Co-precipitation of FtsH2 and AtCHIP with protein extracts from high-intensity light-treated plants. WT, wild-type; lanes 1–3, protein extracts from the *AtCHIP*-over-expressing plant O3486. The input-1 lane was loaded with 50 μg of leaf membrane proteins directly, and the input-2 lane was loaded with FtsH2 precipitated from 500 μg of soluble proteins by 5 μl of FtsH2 antibodies. AtCHIP antibodies were used to pull down FtsH2, and FtsH2 antibodies were used in the Western blot.

during import into the chloroplast. *AtCHIP* over-expression also appears to increase the AtCHIP–FtsH2 interaction under high-intensity light conditions, because more FtsH2 proteins were pulled down by AtCHIP antibodies from *AtCHIP* over-expressing plants when protein extracts from
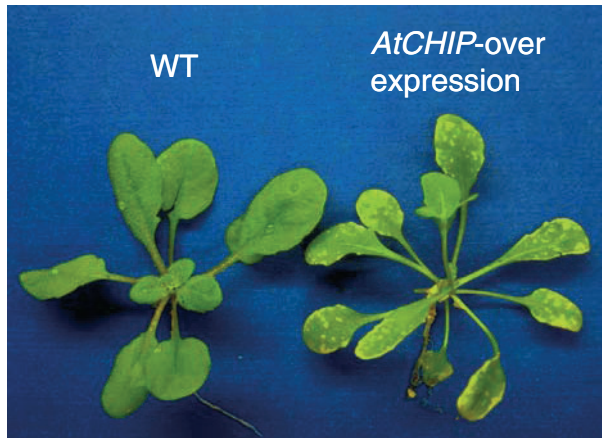
Figure 5. Phenotypes of a wild-type plant and an *AtCHIP*-over-expressing plant after high-intensity light treatment. WT, wild-type plant; *AtCHIP* over-expression, an *AtCHIP*-over-expressing plant (i.e. O3486) that displays a severe necrosis phenotype. The high-intensity light treatment consisted of 22 h under normal light (150 μmol photons m^{-2} sec^{-1}) and 2 h under high-intensity light (1200 μmol photons m^{-2} sec^{-1}) per day for 1 week.

Figure 6. Steady-state levels of D1 protein and its damaged product in wild-type and *AtCHIP*-over-expressing plants after high-intensity light treatment.
(a) Western blot analysis of the steady-state levels of D1 and the 23 kDa fragment in wild-type and five *AtCHIP*-over-expressing plants after high-intensity light treatment.
(b) Relative levels of the full-length D1 in wild-type and *AtCHIP*-over-expressing plants (obtained by analyzing the levels of D1 normalized to the levels of APX3 shown in (a) using densitometry analysis).
(c) Relative levels of the 23 kDa fragment in wild-type and *AtCHIP*-over-expressing plants (obtained by analyzing the levels of the 23 kDa fragment normalized to the levels of APX3 shown in (a) using densitometry analysis). WT, wild-type; lanes 1–5, five independent *AtCHIP*-over-expressing plants. APX3, ascorbate peroxidase 3.

high-intensity light-treated plants were used in the co-precipitation experiment (comparing lane 1 to lane WT in Figure 4e).

AtCHIP-over-expressing plants display a cell-death phenotype under high-intensity light conditions

Because the chloroplast protease FtsH has been implicated as playing a protective role under high-intensity light conditions (Bailey *et al.*, 2002; Lindhal *et al.*, 2000; Sakamoto *et al.*, 2003), we analyzed the phenotype of *AtCHIP*-over-expressing plants under high-intensity light conditions. After treating plants with a light intensity of 1200 μmol photons m^{-2} sec^{-1} for 2 h per day for a week, we observed a cell-death phenotype (i.e. necrosis) on the leaves of *AtCHIP*-over-expressing plants (Figure 5), but not on wild-type leaves. The cell-death phenotype observed on the leaves of *AtCHIP*-over-expressing plants is probably due to reduced steady-state level of FtsH1, and plants with lower level of FtsH1 tend to have more severe necrosis. For example, the *AtCHIP*-over-expressing plant O3486 has the lowest level of FtsH1 (Figure 2), and displays the most severe necrosis phenotype under high-intensity light conditions (Figure 5).

High-intensity light-induced cell death is probably due to decreased capacity to remove photodamaged D1 protein in the PSII of AtCHIP-*over-expressing plants*

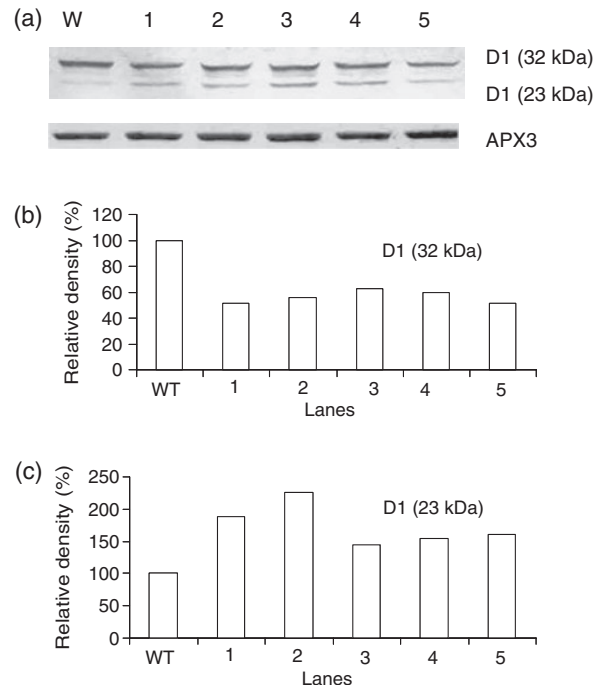Under high-intensity light conditions, the PSII reaction center protein D1 is very prone to photodamage (Kyle *et al.*, 1984; Mattoo *et al.*, 1984), which demands a highly efficient repair system to replace the damaged D1 in the PSII. This repair system can detect the damaged D1 protein and remove it from PSII rather efficiently under high-intensity light conditions (Barber and Andersson, 1992; Melis, 1999). Newly synthesized D1 protein can then be inserted into the PSII reaction center. Previous studies have suggested that the damaged D1 is removed by FtsH protease in chloroplast thylakoid membranes (Bailey *et al.*, 2002; Lindhal *et al.*, 2000; Sakamoto *et al.*, 2003). It appears that the damaged D1 is first cleaved into two fragments, the 23 and 10 kDa fragments, by Deg2 or an unknown protease (Haußühl *et al.*, 2001; Huesgen *et al.*, 2005, 2006), then FtsH protease degrades the 23 kDa fragment (Lindhal *et al.*, 2000). We analyzed the steady-state level of D1 protein in *AtCHIP*-over-expressing plants by Western blot analysis. Interestingly, we observed a decrease in the steady-state level of the full-length D1, but an increase in the 23 kDa fragment of D1 in *AtCHIP*-over-expressing plants after high-intensity light treatment

(Figure 6a). The increase in the 23 kDa fragment of D1 is probably due to a decreased level of FtsH protease in *AtCHIP*-over-expressing plants, which leads to inefficient removal of the damaged D1 fragment from thylakoid membranes. The steady-state level of another chloroplast protease, Deg2, did not differ between wild-type and *AtCHIP*-over-expressing plants after high-intensity light treatment (data not shown).

Cell death observed in the leaves of AtCHIP-*over-expressing plants is associated with increased production of reactive oxygen species*

The cell death in the leaves of *AtCHIP*-over-expressing plants resembles the hypersensitive reaction that occurs during an incompatible interaction between an avirulent pathogen and a resistant host. The hypersensitive reaction is a resistance response that leads to localized cell death in and near the pathogen infection site, which limits further infection by the pathogen in the host plant (Levine *et al.*, 1994). Seo *et al.* (2000) reported that reduced levels of FtsH protease in tobacco mosaic virus-infected tobacco leaves accelerate the hypersensitive reaction, which is similar to our case where reduced FtsH1 is associated with the production of necrosis under high-intensity light conditions. As reactive oxygen species have been shown to be involved in the cell death observed in disease lesion-mimic mutants (Jabs *et al.*, 1996), they are probably the agents that kill plant cells. To test whether reactive oxygen species are involved in the cell death of *AtCHIP*-over-expressing plants, we examined the production of H_2O_2 in both wild-type plants and *AtCHIP*-over-expressing plants. Plant leaves were infiltrated with 3,3′-diaminobenzidine (DAB) in the presence or absence of ascorbate. If H_2O_2 is produced in the dying cells, it will interact with DAB to produce a dark-brown or reddish pigment deposit. However, the dark-brown or reddish pigment can be prevented if ascorbate is provided (Thordal-Christensen *et al.*, 1997). All *AtCHIP*-over-expressing plants that display necrosis showed strong staining with DAB (Figure 7), but no DAB staining was observed in any of our wild-type control plants. The DAB staining was largely prevented if ascorbate was provided (Figure 7), indicating that DAB staining was due to the production of H_2O_2 in *AtCHIP*-over-expressing plants.

Reduced expression of FtsH1 *by antisense suppression in Arabidopsis also leads to sensitivity to high-intensity light treatment*

To further test whether there is a causal relationship between the reduced level of FtsH1 and the cell death under high-intensity light conditions, we created *FtsH1* antisense plants. Of the 40 independent transgenic antisense plants, most appear to have just one T-DNA
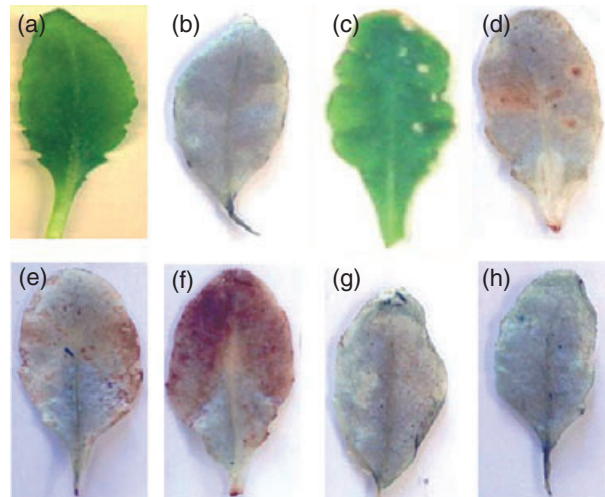

Figure 7. Localized cell death on the leaves of *AtCHIP*-over-expressing plants is associated with the production of H_2O_2.
The reddish pigment is the product of DAB oxidization by H_2O_2.
(a) Wild-type leaf after high-intensity light treatment;
(b) wild-type leaf after high-intensity light treatment followed by DAB treatment;
(c) *AtCHIP*-over-expressing leaf after high-intensity light treatment;
(d)–(f) three *AtCHIP*-over-expressing leaves after high-intensity light treatment followed by DAB treatment;
(g) and (h) two leaves from *AtCHIP*-over-expressing plants after high-intensity light treatment followed by DAB treatment in the presence of ascorbate.

insertion (Table 1) based on the segregation data for kanamycin resistance versus sensitivity of their progeny. To test whether these antisense plants express *FtsH1* antisense transcripts, we conducted RNA blot analysis. Because a double-stranded cDNA of *FtsH1* was used as the probe in hybridization, it recognizes both the endogenous *FtsH1* transcript and the *FtsH1* antisense transcript in the RNA blot analysis (Figure 8a). Therefore, the increased band intensity from antisense plants is most likely due to the presence of *FtsH1* antisense transcript (Figure 8a). The consequence of antisense suppression was clearly revealed by Western blot analysis, which showed significantly reduced steady-state levels of FtsH1 protein in all antisense plants tested (Figure 8b,c). Furthermore, the steady-state level of FtsH2 was also reduced in FtsH1 antisense plants (Figure 8d,e). The reduction in the steady-state level of FtsH2 might be due to interference from the *FtsH1* antisense transcript or the concomitant reduction in steady-state levels of other FtsH subunits in the FtsH protease complex. The outcome of these reductions in FtsH subunits is probably reduced FtsH enzyme activity in the chloroplast. We not only observed the cell-death (necrosis) phenotype among the majority of antisense plants that were subjected to high-intensity light treatment (Figure 9), but also observed a chlorosis phenotype under normal growth conditions in the same group

Table 1 Segregation data and phenotypes of the *FtsH1* antisense plants

Transgenic line	Km^R	Km^S	Probability of one T-DNA insertion[a]	Chlorosis[b]	Necrosis[c]
WT	0	87		–	–
FA11	82	23	>0.45	+	+
FA12	94	30	>0.80	+	+
FA13	80	24	>0.65	+	+
FA14	90	35	>0.40	+	+
FA15	67	20	>0.65	+	+
FA16	66	24	>0.70	+	+
FA17	60	24	>0.45	+	+
FA26	89	25	>0.45	–	–
FA29	92	35	>0.50	+	+
FA30	88	31	>0.80	+	–
FA31	104	33	>0.80	+	+
FA32	132	42	>0.80	–	NT
FA33	87	26	>0.60	+	NT
FA34	98	30	>0.65	+	NT
FA35	79	26	>0.95	+	NT
FA36	96	34	>0.75	+	NT
FA37	112	41	>0.60	+	NT
FA38	134	43	>0.80	+	NT
FA39	86	27	>0.75	+	NT
FA40	88	32	>0.60	+	NT

Km^R, kanamycin-resistant; Km^S, kanamycin-sensitive; NT, not tested.
[a]Probability that only one T-DNA was inserted, based on chi-squared analysis of segregation data.
[b]The appearance of chlorosis at rosette stage was scored as a plus.
[c]The appearance of cell death after high-intensity light treatment was scored as a plus.

of transgenic plants (Figure 9). There is clearly a causal relationship between reduced expression of *FtsH1* and the cell-death or chlorosis phenotype in the antisense plants.

AtCHIP and FtsH1 interact with the cytosolic Hsp70

Animal CHIP proteins are chaperone co-factors that bind to Hsp70/Hsp90 and regulate their activities (Murata *et al.*, 2001). Therefore, we tested whether AtCHIP interacts with the Arabidopsis Hsp70 by conducting co-precipitation experiments. In Arabidopsis, there are five highly conserved cytosolic Hsp70 proteins, of which Hsc70-1, Hsc70-2 and Hsc70-3 are present under non-stress conditions (Sung *et al.*, 2001). A monoclonal antibody has been developed that binds specifically to the Arabidopsis cytosolic Hsp70 proteins, but not to any other Hsp70 proteins in Arabidopsis (Anderson *et al.*, 1994). We used this monoclonal antibody in co-precipitation experiments. As expected, AtCHIP antibodies pulled down a protein with a molecular weight of 70 kDa that is recognized by the Hsp70 monoclonal antibody (Figure 10a). The AtCHIP antibodies pulled down more Hsp70 from leaf cellular
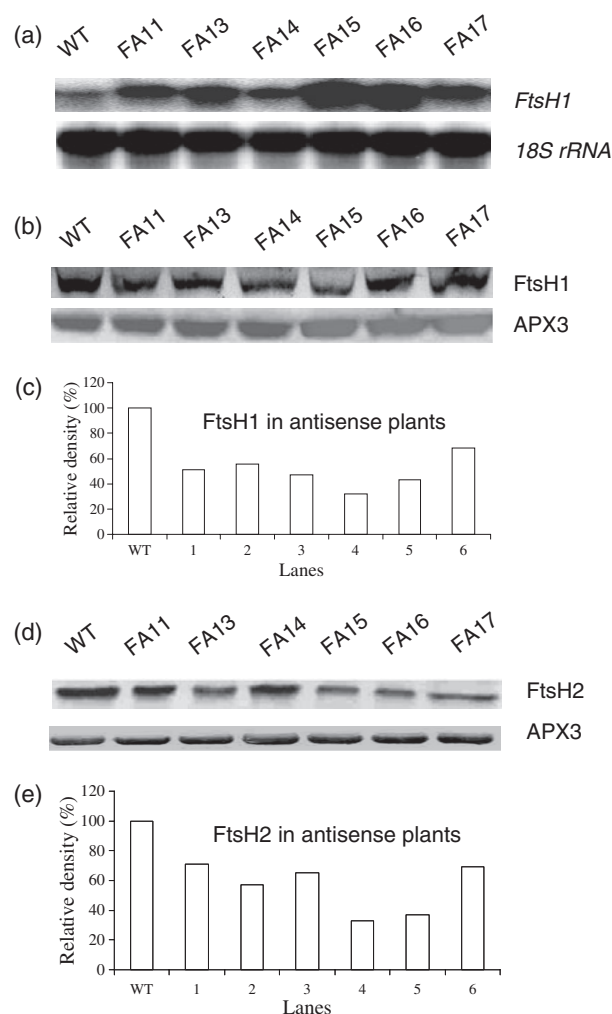
Figure 8. Northern and Western blot analyses of *FtsH1* antisense plants.
(a) Northern blot analysis. WT, wild-type; FA11–FA17, six independent *FtsH1* antisense plants. The genes used as probes are listed on the right (18S rRNA is the RNA loading control).
(b) Western blot analysis of FtsH1 in *FtsH1* antisense plants. FtsH1 and APX3 antibodies were used.
(c) Relative levels of FtsH1 in wild-type and *FtsH1* antisense plants (obtained by analyzing the steady-state levels of FtsH1 normalized to the steady-state levels of APX3 shown in (b) using densitometry analysis).
(d) Western blot analysis of FtsH2 in *FtsH1* antisense plants. FtsH2 and APX3 antibodies were used.
(e) Relative levels of FtsH2 in wild-type and *FtsH1* antisense plants (obtained by analyzing the steady-state levels of FtsH2 normalized to the steady-state levels of APX3 shown in (d) using densitometry analysis). WT, wild-type; lanes 1–6, six independent *FtsH1* antisense plants, i.e. FA11–FA17, respectively. APX3, ascorbate peroxidase 3.

extracts of *AtCHIP*-over-expressing plants treated with high-intensity light (compare lane 1 to lane WT in Figure 10b), similar to the co-precipitation results with AtCHIP and FtsH1 or FtsH2 (Figures 1c and 4e). As chloroplast-targeted proteins are usually bound by Hsp70 before they are imported into the chloroplast, we predicted that FtsH1 would interact with Hsp70 *in vivo*.
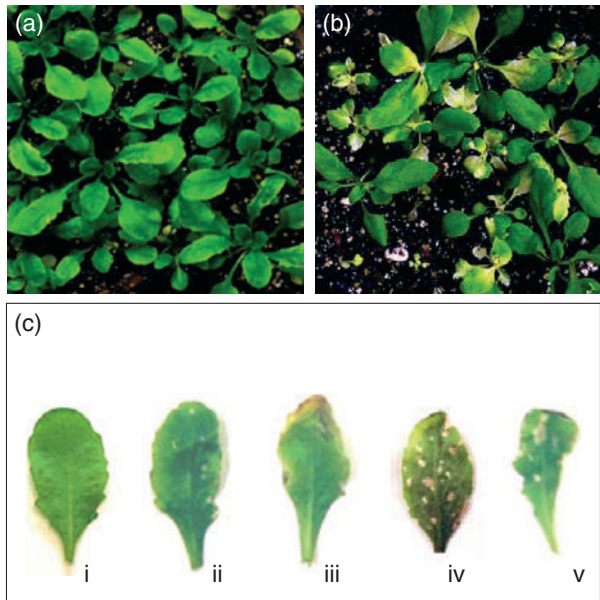
Figure 9. Phenotypes of wild-type and *FtsH1* antisense plants under normal growth conditions and after high-intensity light treatment.
(a) Wild-type plants under normal growth conditions.
(b) *FtsH1* antisense plants under normal growth conditions.
(c) Wild-type (i) and *FtsH1*-antisense plants (ii–v) after high-intensity light treatment.

As expected, our co-precipitation experiments indicated that FtsH1 antibodies could pull down Hsp70 very efficiently (Figure 10c). Furthermore, more Hsp70 proteins were pulled down if high-intensity light-treated *AtCHIP*-over-expressing plants were used in the co-precipitation experiments (Figure 10d).

The steady-state level of cytosolic Hsp70 is increased in AtCHIP-over-expressing plants under high-intensity light conditions

High-intensity light appears to increase the protein–protein interaction between any pair of the three proteins in our study (AtCHIP, FtsH1 and Hsp70), because the amount of co-precipitated protein is always high with cellular extracts from high-intensity light-treated plants. This suggests the possibility that the steady-state level of Hsp70 might be affected by high-intensity light itself. Our RNA blot data indicated that the transcript level of the cytosolic Hsp70 was significantly increased by high-intensity light treatment in *AtCHIP*-over-expressing plants (Figure 11a), which in turn led to an increase in the steady-state level of Hsp70 protein as shown in our Western blot analysis (Figure 11b), although the increase in the steady-state level of Hsp70 protein varied from 38–92% higher than that in wild-type plants under high-intensity light conditions (Figure 11c).
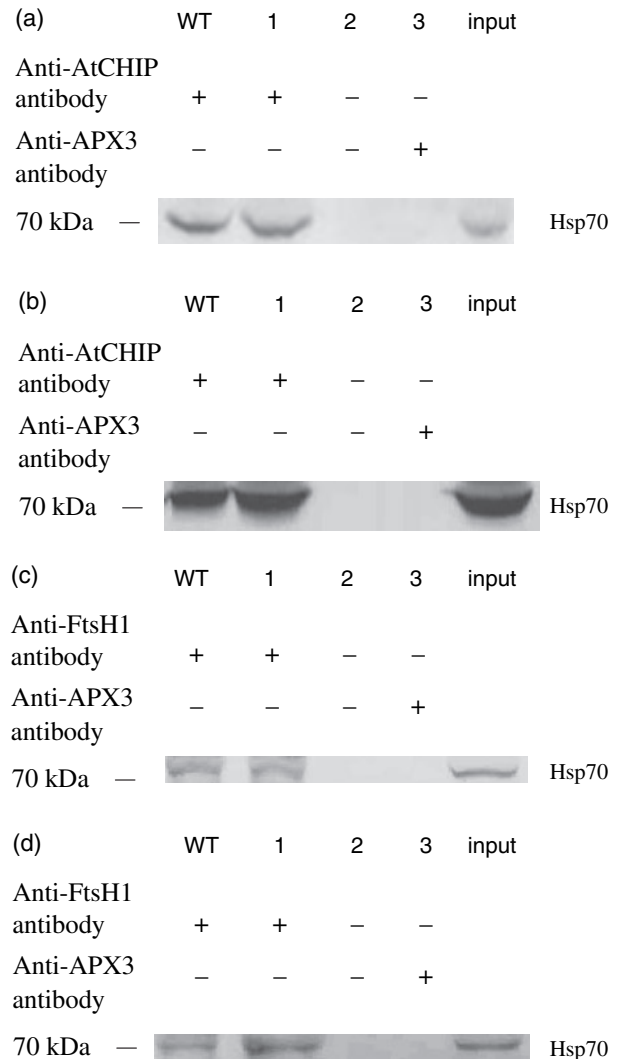

Figure 10. Co-precipitation of AtCHIP with Hsp70 and FtsH1 with Hsp70 in plant cells.
(a) Co-precipitation of AtCHIP and Hsp70 with soluble protein extracts from normal light-treated plants. AtCHIP antibodies were used to pull down Hsp70, and the Hsp70 monoclonal antibody was used in the Western blot.
(b) Co-precipitation of AtCHIP and Hsp70 with soluble protein extracts from high-intensity light-treated plants.
(c) Co-precipitation of FtsH1 and Hsp70 with soluble protein extracts from normal light-treated plants. FtsH1 antibodies were used to pull down Hsp70, and the monoclonal Hsp70 antibody was used in the Western blot.
(d) Co-precipitation of FtsH1 and Hsp70 with soluble protein extracts from high-intensity light-treated plants. WT, wild-type; lanes 1–3, protein extracts from the *AtCHIP*-over-expressing plant line O3486. The input lane was loaded with 50 μg of leaf soluble proteins.

Discussion

Our data indicate that AtCHIP ubiquitylates FtsH1 *in vitro* (Figure 1a) and interacts with FtsH1 and FtsH2 *in vivo* (Figures 1b and 4d). Furthermore, *AtCHIP* over-expression leads to decreased steady-state levels of FtsH1 and FtsH2 (Figures 2c and 4b), localized leaf cell death (Figure 5), and
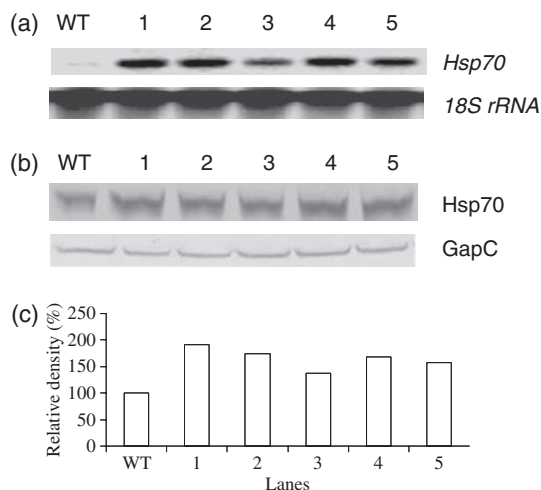
Figure 11. Steady-state levels of the transcript and protein of cytosolic Hsp70 in wild-type and *AtCHIP*-over-expressing plants after high-intensity light treatment.
(a) RNA blot analysis of the *Hsp70* transcript after high-intensity light treatment. The blots were probed with labeled cDNAs for *Hsp70* and 18S rRNA. The 18S rRNA is used as the RNA loading control.
(b) Western blot analysis of the steady-state level of Hsp70 after high-intensity light treatment. Hsp70 monoclonal antibody and GapC antibodies were used in the Western blot experiments. GapC, cytosolic glyceraldehyde-3-phosphate-dehydrogenase (used as the loading control for the Western blot).
(c) Relative levels of the cytosolic Hsp70 in wild-type and five *AtCHIP*-over-expressing plants after high-intensity light treatment (obtained by analyzing the levels of Hsp70 normalized to the levels of GapC shown in (b) using densitometry analysis). WT, wild-type; lanes 1–5, five independent *AtCHIP*-over-expressing plants, i.e. O3261, O3486, O264, O3122 and O476, respectively.

Figure 12. Model of how cell death occurs on the leaves of *AtCHIP*-over-expressing plants under high-intensity light conditions.
The high-intensity light damages the 32 kDa D1 protein in the thylakoid membranes, then the damaged D1 is cleaved into two fragments, 23 and 10 kDa, by thylakoid membrane-associated Deg2 protease or an unknown protease(s). Because *AtCHIP* over-expression causes the degradation of FtsH1 and FtsH2 under high-intensity light conditions, this leads to the inefficient removal of the damaged D1 23 kDa fragment, from the thylakoid membranes. The lack of repair of PSII leads to the over-production of H_2O_2 under high-intensity light conditions, which kills the leaf cells.

accumulation of a fragment of the FtsH substrate protein D1 under high-intensity light conditions (Figure 6). The cell death is probably caused by over-production of H_2O_2 in the *AtCHIP*-over-expressing plants (Figure 7), which is a result of the decreased capacity of FtsH protease to remove the photodamaged D1 protein under high-intensity light conditions. Our data strongly suggest that FtsH1 (and possibly FtsH2 as well) is a substrate of AtCHIP *in vivo*, and there is a causal relationship between increased expression of *AtCHIP* and the decreased FtsH protease level in chloroplasts under high-intensity light conditions. A cell-death phenotype was also observed in *FtsH1* antisense plants that were subjected to high-intensity light treatment (Figure 9), confirming our prediction that a decrease in the FtsH protease activity could lead to cell death under high-intensity light conditions. Therefore, the molecular events that lead to the cell death in *AtCHIP*-over-expressing plants may be interpreted in the following way. As the PSII reaction center protein D1 is very sensitive to high-intensity light (Kyle *et al.*, 1984; Mattoo *et al.*, 1984), it is damaged under high-intensity light conditions. The damaged D1 is then recognized by the repair system and cleaved into smaller fragments by proteases on both sides of the thylakoid membrane, and these fragments are removed by FtsH protease (Kapri-Pardes *et al.*, 2007).
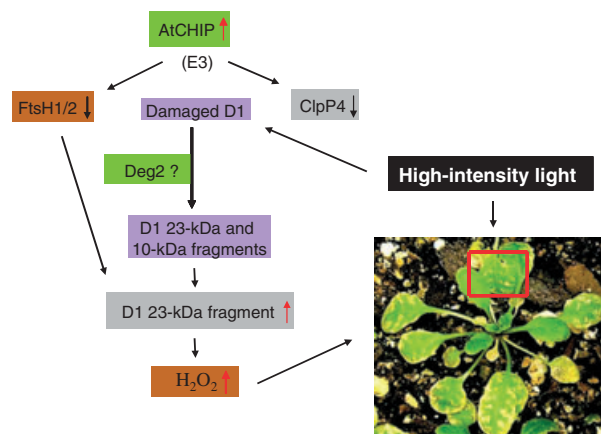
However, in *AtCHIP*-over-expressing plants, the FtsH protease level is reduced because of the *AtCHIP* over-expression, the damaged D1 product (i.e. the 23 kDa fragment) can no longer be removed efficiently, and no new D1 protein can be inserted into the PSII reaction center, leading to the over-production of reactive oxygen species such as H_2O_2 under high-intensity light conditions, which kill leaf cells in *AtCHIP*-over-expressing plants (Figure 12).

Our knowledge about protein refolding and degradation in the eukaryotic cytoplasm has been growing exponentially over the last 20 years, and many molecular chaperones and components of the ubiquitylation system and the 26S proteasome system have been discovered, and the regulatory mechanisms have been under intensive investigation (Ciechanover *et al.*, 2000; Smalle and Vierstra, 2004). However, in comparison with the rich knowledge regarding protein quality control in the cytoplasm, very little is known about protein degradation in organelles. For example, chloroplasts harbor thousands of proteins and enzymes that perform essential biological functions such as photosynthesis and the biosynthesis of fatty acids and many other important compounds in plants, but how those proteins and enzymes are turned over in the chloroplast is not understood. Recently, several protease families such as stroma-located Clp proteases and thylakoid membrane-bound FtsH proteases have been identified (Adam and Clarke, 2002; Adam *et al.*, 2006; Sakamoto, 2006), but little is known about the way these proteins are regulated under normal and stress conditions and the identity of their substrates. Our findings regarding AtCHIP suggest that it

may affect protein quality control in chloroplasts by limiting the availability of components of the chloroplast proteolytic machinery. In Arabidopsis, there are 12 nuclear genes encoding FtsH proteins, of which nine are targeted to chloroplasts. To date, only four subunits have been identified in thylakoid membranes, namely FtsH1, FtsH2, FtsH5 and FtsH8 (Sinvany-Villalobo *et al.*, 2004; Yu *et al.*, 2004), which form hetero-oligomeric hexamers (Adam *et al.*, 2006; Sakamoto *et al.*, 2003; Yu *et al.*, 2004; Zaltsman *et al.*, 2005). The targeting of the precursors of proteolytic subunits of chloroplast proteases for ubiquitylation and degradation by AtCHIP may represent a novel mechanism by which ubiquitylation events in the cytoplasm could regulate protein degradation in the chloroplast, which implies that CHIP is not only a critical regulator of protein quality control in the cytoplasm, but also a critical regulator of protein degradation in the chloroplast. It is not clear why plants use the AtCHIP-mediated protein degradation pathway to down-regulate chloroplast protein degradation, because it would seem to be more economical to exert control at the transcriptional or translational level rather than at the post-translational level. Although there are no apparent differences in the transcript level of *FtsH1* between wild-type and *AtCHIP*-over-expressing plants under high-intensity light conditions (Figure 3), we cannot rule out the possibility that translation of the FtsH1 transcript might be regulated under high-intensity light conditions. As no mechanism has been identified that regulates the activities of chloroplast proteases inside the chloroplast, it is perhaps important to downregulate chloroplast protein degradation under certain stress conditions, and degradation of chloroplast protease precursors that have already been synthesized may be more effective and faster than regulation at transcriptional or translational level.

Although we do not know whether other FtsH subunits are also substrate proteins for AtCHIP *in vivo*, the high sequence similarities between FtsH1 and other FtsH subunits, especially FtsH5 (Sakamoto *et al.*, 2003; Yu *et al.*, 2004), suggest that they are probably also substrate proteins for AtCHIP. Otherwise, it would be difficult to explain why a knockout mutant for FtsH1, i.e. *ftsh1*, does not show an altered phenotype (Sakamoto *et al.*, 2003). The loss of FtsH1 alone may not be detrimental to FtsH activity in the chloroplast, because its closest homolog FtsH5 might compensate for the loss of FtsH1 in the *ftsh1* mutant. However, if two FtsH subunits are missing, or one of the two more abundant FtsH subunits (i.e. FtsH2 or FtsH5) is missing, mutant phenotypes will appear (Sakamoto, 2006; Zaltsman *et al.*, 2005). *AtCHIP*-over-expressing plants have reduced capacity to repair the damage caused by high-intensity light, indicating that two or more FtsH proteolytic subunits are probably reduced in *AtCHIP*-over-expressing plants under high-intensity light conditions. Our data support this hypothesis because the steady-state levels of both FtsH1 and FtsH2 are clearly

reduced in *AtCHIP*-over-expressing plants under high-intensity light conditions (Figures 2c and 4b). An interesting feature about *FtsH1* antisense plants is that they display a chlorosis phenotype under normal growth conditions, which is different from the variegated phenotype of the two FtsH mutants *var1* and *var2* (because of mutations in *FtsH5* and *FtsH2*, respectively) (Sakamoto, 2002). Furthermore, *var1* and *var2* mutants display variegated chlorosis at earlier developmental stages. For example, the first true leaf of *var2* is mostly white (Adam *et al.*, 2005), whereas FtsH1 antisense plants usually display the chlorosis phenotype after the third or the fourth true leaf. The molecular mechanism for the variegated phenotype of *var1* and *var2* mutants is not known (Sakamoto, 2006), although an interesting 'threshold' model was proposed by Yu *et al.* (2004). However, the 'threshold' model based on analyzing *var1* and *var2* mutants cannot be used to explain the more uniform chlorosis phenotype displayed by *FtsH1* antisense plants, which is clearly not caused by the reduced level of FtsH1 alone; instead it is the outcome of a reduced level of FtsH enzyme activity. The reduced level of FtsH2 in the *FtsH1* antisense plants (Figure 8d) probably also contributes to the chlorosis phenotype. Furthermore, as FtsH1 and FtsH5 share high sequence similarity at the DNA level, the *FtsH1* antisense transcript might lead to reduced transcript levels of both FtsH1 and FtsH5 in *FtsH1* antisense plants. This possibility can be studied when isoform-specific antibodies that can distinguish FtsH1 and FtsH5 become available in the future.

Because the degradation of FtsH1 is not increased under normal and heat-stress conditions in *AtCHIP*-over-expressing plants when compared to that in wild-type plants, other component(s) might be the rate-limiting factor(s) in its ubiquitylation and degradation under high-intensity light conditions. The steady-state level of the cytosolic Hsp70 is increased under high-intensity light conditions, suggesting that Hsp70 might be the rate-limiting factor. Nuclear-encoded chloroplast proteins are imported into the chloroplast in an unfolded conformation (Bédard and Jarvis, 2005; Kessler and Schnell, 2006), and, during this process, cytosolic Hsp70 chaperones are probably involved in maintaining unfolded precursors in a transport-competent state (Jackson-Constan *et al.*, 2001; Soll and Schleiff, 2004). As CHIP was initially identified as a chaperone co-factor that regulates the activities of Hsp70 (Ballinger *et al.*, 1999), and CHIP can target the substrates of Hsp70 to the 26S proteasome for degradation (Höhfeld *et al.*, 2001; Murata *et al.*, 2001), it would not be surprising if AtCHIP binds to and ubiquitylates the substrate proteins of Hsp70, such as the FtsH1 precursor, in the cytoplasm prior to the import of FtsH1 into the chloroplast. Targeting of the FtsH1 precursor for ubiquitylation and degradation by AtCHIP provides a cytosolic control over the amount of FtsH1 precursor available for chloroplast import. By

controlling the amount of precursors of protease subunits imported into chloroplasts, AtCHIP might affect the amount of functional proteases in the stroma and in the thylakoid membranes. Therefore, further study on the role of AtCHIP in protein quality control in both cytoplasm and organelles will significantly improve our understanding of protein homeostasis in plant cellular metabolism under normal and stressful conditions.

Experimental procedures

Identification of FtsH1 as an AtCHIP-interacting protein

FtsH1 was identified as a AtCHIP-interacting proteins by a yeast two-hybrid screen (Luo *et al.*, 2006), and matches perfectly the primary sequence of FtsH1 characterized by Lindahl *et al.* (1996). The gene identification number for FtsH1 is At1g50250.

Expression of FtsH1 in bacterial cells

A partial *FtsH1* fragment that encodes residues 225–716 (immediately after the second transmembrane domain) was amplified from a cDNA library using primers FtsH-2 and FtsH-7, and then cloned into the pET-30b vector (Novagen; http://www.emdbiosciences.com) using restriction enzymes *Xho*I and *Sma*I. Recombinant vectors were introduced into the bacterial strain BL21(DE3) for expression in the presence of kanamycin. His-tagged FtsH1 was purified according to the manufacturer's protocol (His-Bind kits, Novagen). The sequences of oligonucleotides FtsH-2 and FtsH-7 are AGCTCTCGAGTCAAGAAATATACAACTCAGCTT and AGCCCCGG-GAAGGCGAGCTCAGGGAGGCCC, respectively.

In vitro ubiquitylation of FtsH1

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 the E2 conjugase, rabbit E1 (Calbiochem; http://www.emdbiosciences.com) as the activase, bovine ubiquitin (Sigma, http://www.sigmaaldrich.com/), and the partial *FtsH1* fragment as the substrate. Polyclonal antibodies against FtsH1 were used in the immunoblot analysis.

Immunoprecipitation

Protein sample preparation. Wild-type Arabidopsis (ecotype C24) and *AtCHIP*-over-expressing seedlings were grown in soil as described previously (Yan *et al.*, 2003). Fresh plant leaf tissues (100 mg) were harvested at about 2 weeks old and ground in liquid nitrogen using a mortar and pestle, and 200 μl of cold extraction buffer (50 mM sodium phosphate buffer, pH 7, 0.1 mM EDTA) was added to the powder. The extraction solution was centrifuged at 13 000 *g* for 10 min at 4°C. The supernatant fraction, which contains mainly the soluble proteins, was used for co-precipitation experiments, whereas the pellet fraction, which contains organelles and membrane proteins, was resuspended into 200 μl of 50 mM MOPS buffer (pH 7.8) with 0.1 mM DTT and 2% Triton X-100. The resuspended pellet was mixed well, and the suspension was centrifuged at 13 000 *g* for 10 min at 4°C, and then the supernatant was transferred to a tube containing 2× protein loading buffer for Western blot experiments. The protein concentration in the sample was determined by the Bradford (1976) method using bovine serum albumin as a standard.

Protein A–agarose slurry preparation. About 50 μl of protein A–agarose slurry (Sigma) was pre-soaked in cold NP-40 buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 1% NP-40) for at least 2 h on ice, then centrifuged at 6000 *g* for 30 sec. The supernatant was removed, and the pellet was washed once with 500 μl of cold NP-40 buffer, then resuspended into 50 μl of cold NP-40 buffer.

Co-precipitation experiment. About 15 μl of AtCHIP antibodies (for experiments shown in Figures 1b,c, 4d,e and 10a,b) or 5 μl of FtsH2 antibodies (for experiments shown in Figure 10c,d) were mixed with 500 μg of protein from Arabidopsis leaf cellular extracts prepared as above in an Eppendorf tube, and incubated on ice for 60 min, then 50 μl of washed protein A–agarose slurry was added, and the mixture was incubated for 1 h at 4°C on a rotary shaker. The mixture was then centrifuged at 6000 *g* for 30 sec at 4°C, and the supernatant was carefully removed. The protein A–agarose slurry was washed five times with 500 μl of cold NP-40 buffer. After the last wash, the agarose–protein complex was collected, and mixed with 2× sodium dodecyl sulfate (SDS) loading buffer (125 mM Tris–Cl, 2% SDS, 20% glycerol, 200 mM dithiothreitol, 0.01% bromophenol blue, pH 6.8), vortexed gently, and then centrifuged at 6000 *g* for 30 sec to remove protein A–agarose beads. Samples were boiled at 100°C for 5 min before loading onto a 12% SDS–polyacrylamide gel for electrophoresis. The input lanes were loaded with 50 μg of total membrane proteins (Figures 1b,c and 4d,e), or FtsH2 protein precipitated by 5 μl of FtsH2 antibodies from 500 μg of soluble proteins (Figure 4d,e), or 50 μg of soluble proteins (Figure 10a,b). After electrophoresis, the proteins were transferred to a nitrocellulose membrane. The membrane was blocked with a milk solution first, incubated with FtsH1, FtsH2, or Hsp70 monoclonal antibodies for 2 h at room temperature, and washed three times (10 min each time) prior to incubation with alkaline phosphatase-conjugated goat anti-rabbit antibodies or alkaline phosphatase-conjugated rabbit antimouse antibodies (Bio-Rad, http://www.bio-rad.com/). The membrane was washed again three times (10 min each time) before being used for color development with alkaline phosphatase substrate solution (Bio-Rad). The blot was then photographed and analyzed.

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; http://www.clorox.com) 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 1 week. 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 1 week. The high-intensity light treatment consisted of 22 h under normal light (150 μmol m^{-2} sec^{-1}) and 2 h under high-intensity light (1200 μmol photos m^{-2} sec^{-1}) per day for 1 week. Plants not treated with stresses were kept under normal growth conditions.

Leaf protein extraction for Western blot 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 × SDS loading buffer. To prepare the membrane protein fraction that contains APX3, D1, FtsH1 and FtsH2, the pellet fraction was resuspended into 200 μl of 50 mM NaHPO$_4$ with 2% Triton X-100 and 200 μl of 2 × SDS buffer. The resuspended pellet was boiled in a water bath for 10 min, and centrifuged at 13 000 g for 10 min at 4°C. The supernatant was transferred to a fresh tube. Protein concentration in the extraction buffer was determined by the Bradford (1976) method using bovine serum albumin as a standard. Proteins from *AtCHIP*-over-expressing plants and wild-type plants were subjected to electrophoresis in a 12% SDS–polyacrylamide gel. Polyclonal D1 antibodies that recognize both full-length D1 protein and the damaged 23 kDa D1 fragment, polyclonal antibodies against APX3, GapC, FtsH1 and FtsH2, and the monoclonal Hsp70 antibody were used in the Western blot experiments. The conditions for blotting and color development were the same as described above. The reason for using APX3 as the loading control for membrane proteins in Western blot experiments (i.e. Figures 2, 4, 6 and 8) was that the level of transcript of *APX3* does not change under various conditions including high-intensity light conditions (Kimura *et al.*, 2003; Narendra *et al.*, 2006).

RNA isolation and hybridization

Total RNA was isolated from Arabidopsis plants using TRIzol reagent (Invitrogen, http://www.invitrogen.com/), 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 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 1 mM EDTA, 40 mM NaHPO$_4$ (pH 7.2) and 1% SDS at 63°C. The same filter was used for hybridizations with the various probes, and the filter was then stripped by washing twice (15 min each) in 2 mM Tris (pH 8.2), 2 mM EDTA (pH 8.0) and 0.1% SDS at 75°C. A full-length cDNA of *Hsp70* and a full-length cDNA for 18S rRNA were used as the probes in Northern blot analyses.

Leaf DAB staining

Wild-type and transgenic leaves were each divided into two groups. One group was infiltrated with incubation buffer [0.05% w/v 3,3'-diaminobenzidine 4-HCL (DAB) and 10 mM MES, pH 6.5)], incubated in the light for 2 h, and then soaked in a mixture of acetic acid, phenol and water (1:1:1, v/v/v) in the dark for 24 h. The leaves were then photographed using a dissecting microscope (Zeiss 4750, http://www.zeiss.com/). The other group was infiltrated with a buffer that contains ascorbate (10 mM), DAB and MES. All subsequent treatments for the second group were the same for as the first group.

Creation of FtsH1 *antisense plants*

The binary vector pBIN121 (Jefferson *et al.*, 1987) was digested with *Xba*I and *Sst*I, and the β-glucuronidase gene sequence was replaced with a full-length *FtsH1* cDNA in the antisense orientation. The *FtsH1* cDNA was isolated from a cDNA library by PCR using oligonucleotides FtsH-5 (AGCTTCTAGATCAAGAAATATACAACTCAGCTT) and FtsH-6 (AGCTGAGCTCATGGCTTCTAACTCATTACTAC). The transformation vector thus created was introduced into wild-type Arabidopsis plants by using the flower dip method described by Clough and Bent (1998).

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