The chloroplastic lipocalin AtCHL prevents lipid peroxidation and protects Arabidopsis against oxidative stress

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SUMMARY

Lipocalins are small ligand-binding proteins with a simple tertiary structure that gives them the ability to bind small, generally hydrophobic, molecules. Recent studies have shown that animal lipocalins play important roles in the regulation of developmental processes and are involved in tolerance to oxidative stress. Plants also possess various types of lipocalins, and bioinformatics analyses have predicted that some lipocalin members may be present in the chloroplast. Here we report the functional characterization of the Arabidopsis thaliana chloroplastic lipocalin AtCHL. Cellular fractionation showed that AtCHL is a thylakoid luminal protein. Drought, high light, paraquat and abscisic acid treatments induce AtCHL transcript and protein accumulation. Under normal growth conditions, knockout (KO) and over-expressing (OEX) lines do not differ from wild-type plants in terms of phenotype and photosynthetic performance. However, KO plants, which do not accumulate AtCHL, show more damage upon photo-oxidative stress induced by drought, high light or paraquat. In contrast, a high level of AtCHL allows OEX plants to cope better with these stress conditions. When exposed to excess light, KO plants display a rapid accumulation of hydroxy fatty acids relative to the wild-type, whereas the lipid peroxidation level remains very low in OEX plants. The increased lipid peroxidation in KO plants is mediated by singlet oxygen and is not correlated with photo-inhibition of the photosystems. This work provides evidence suggesting that AtCHL is involved in the protection of thylakoidal membrane lipids against reactive oxygen species, especially singlet oxygen, produced in excess light.

Keywords: lipocalin, stress tolerance, lipid peroxidation, chloroplast, thylakoid lumen, paraquat.

INTRODUCTION

Lipocalins are small ligand-binding proteins found in bacteria and in invertebrate and vertebrate animals (Sanchez et al., 2006a). They have a simple tertiary structure that gives them the ability to bind small, usually hydrophobic, molecules. Animal lipocalins play important roles in the regulation of immunological and developmental processes, and are involved in responses to various stress factors and in signal transduction pathways. The insect glial lipocalin Lazarillo has a protective role against oxidative stress conditions, and its absence increases lipid peroxidation and reduces life span in the fruit fly Drosophila melanogaster (Sanchez et al., 2006b). In contrast, its over-expression protects the fruit fly against the effects of starvation, hypoxia and hyperoxia, and increases the fly’s life span (Walker et al., 2006). Given the major roles played by these proteins in animal systems, it is important to determine whether they are present in plant systems and what functions they may have. As part of our efforts to identify abiotic stress-induced genes, we have cloned and analyzed plant proteins that are members of the classic lipocalin family (Frenette Charron et al., 2002).

Data mining of genomic databases and bioinformatics predictions indicated that many plants possess lipocalins, and these proteins were classified as temperature-induced lipocalins (TILs) and chloroplastic lipocalins (CHLs) (Charron et al., 2005). Sequence, structure and phylogenetic analyses revealed that these proteins share homology with three evolutionarily related lipocalins: bacterial lipocalin Blc, mammalian apolipoprotein D, and the insect protein Lazarillo. Expression of the wheat TaCHL gene, which encodes a
chloroplast-associated true lipocalin, is induced by low temperature, and its level of expression is associated with the plant’s capacity to develop freezing tolerance. The encoded protein shares 25% identity and 35% similarity with the wheat plasma membrane-associated TaTIL-1 lipocalin. A major difference is the presence of a predicted transit peptide at the N-terminal end of TaCHL, suggesting that it probably accumulates in the chloroplast.

Despite the accumulating knowledge on the molecular features of plant lipocalins, little is known about their function at the cellular and biochemical levels. To help determine the function of plant lipocalins, we have isolated genes encoding homologous proteins in the model plant Arabidopsis thaliana. Using reverse genetic approaches, we recently showed that the Arabidopsis plasma membrane-associated protein AtTIL is involved in modulating tolerance to oxidative and freezing stresses (Charron et al., 2008). AtTIL knockout plants are very sensitive to paraquat treatment, and etiolated plants die shortly after transfer to light. They also accumulate high levels of hydrogen peroxide and other reactive oxygen species (ROS). Conversely, AtTIL over-expression enhances tolerance to freezing and paraquat treatments, and to light stress. It was recently shown that deletion of AtTIL also affects the tolerance of Arabidopsis to heat stress (Chi et al., 2009).

The homology between the AtTIL and AtCHL proteins and the fact that the corresponding wheat genes are induced by stresses suggest that the two proteins could share a similar function at the cellular level, albeit in different compartments. The predicted localization of CHLs in the chloroplasts and their accumulation in response to temperature stress in wheat support the hypothesis that these proteins might act as scavengers of potentially harmful molecules induced by temperature stress and excess light. In this work, we characterized knockout, complementation and over-expression Arabidopsis lines to determine the cellular and biochemical functions of AtCHL.

RESULTS

AtCHL is localized in the thylakoid lumen

Homology-based searches enabled us to identify and isolate the wheat lipocalin gene TaCHL (ABB02411) and its orthologue AtCHL from Arabidopsis (CAB41869). Bioinformatics analyses using TargetP and ChloroP predicted that the CHLs have an N-terminal transit peptide that targets the proteins to the chloroplasts. To confirm this prediction, intact chloroplasts were isolated and fractionated into their various compartments (Figure 1a). Immunoblot analysis revealed that AtCHL is specifically localized in the thylakoid lumen (Figure 1b). The integrity and purity of the various fractions were assessed by immunoblot analyses using antibodies recognizing proteins that are known to accumulate in the lumen of the thylakoids [plastocyanin (PC)], in the thylakoid membranes [light-harvesting complex IIb (LhcIIb)] or in the stroma [ribulose biphosphate carboxylase oxygenase small subunit (RbcS)]. The data confirmed the purity of the sub-organelar fractions (Figure 1b). Lumenal proteins can be imported in the thylakoid lumen via the Tat (twin-arginine transport) pathway; however, the AtCHL sequence does not show a lumenal targeting motif such as that determined by Peltier et al. (2000). This suggests that import into the lumen probably occurs via interactions with other proteins.

Effect of abiotic stress treatments on AtCHL accumulation

We investigated the accumulation of AtCHL mRNA and protein in soluble extracts prepared from aerial parts of wild-type (WT) Col-0 plants subjected to various stress conditions. The data show that the AtCHL mRNA and protein levels are low when plants are grown in soil under normal, control conditions (CTRL; Figure 2a,b). Dehydration stress strongly increases AtCHL mRNA and protein levels. Treatment with the oxidant paraquat or high light also increases AtCHL expression, but to a lesser extent than dehydration, while NaCl (salinity) and heat shock treatments have no effect. Time-course studies indicated that accumulation of both the transcript and protein is rapid following paraquat (oxidative stress) and dehydration treatments (Figure 2c,d). Application of abscisic acid (ABA), the hormone that shows the closest association with dehydration stress, only has a moderate effect on AtCHL expression. Interestingly, a low-
temperature treatment increased the mRNA level but not the protein level (Figure 2a,b). Expression of wheat TaCHL was also shown to be induced by cold, but protein accumulation was not determined (Charron et al., 2005).

AtCHL increases tolerance towards oxidative stress induced by paraquat or drought treatment

To determine the function of AtCHL, we characterized lines that under- or over-express the AtCHL gene. Only one T-DNA insertion line for AtCHL (SALK_133049) was available in the SALK collection (Alonso et al., 2003), and was obtained from the Arabidopsis Biological Resource Center (Ohio State University). This line carries an insertion at the 5′ end of the first exon of the AtCHL gene (At3g47860) (Figure 3a). As shown in Figure 3(b), WT Col-0 plants show a low level of AtCHL protein accumulation in a total soluble extract from aerial parts of the plant, while plants of the SALK line show no detectable

Figure 2. Effect of abiotic stresses on the expression of AtCHL.
(a) AtCHL transcript accumulation in leaf extracts of soil-grown WT Col-0 plants after various stress treatments. Total RNA was extracted from aerial parts of the plants, and mRNA was detected by RT-PCR. Actin mRNA was used as an internal control.
(b) AtCHL protein level in soil-grown plants after various stress treatments. Top panel: immunoblot analysis using the rabbit polyclonal anti-AtCHL antibody. Bottom panel: Coomassie brilliant blue-stained RuBisCO band shown as a load control.
(c) AtCHL transcript accumulation in leaf extracts of plate-grown WT Col-0 plants before (0 h) and after 3 or 6 h of short stress treatments. Total RNA was extracted from aerial parts of the plants, and mRNA was detected by RT-PCR. Actin mRNA was used as an internal control.
(d) AtCHL protein level after various stress treatments. Top panel: immunoblot analysis using the rabbit polyclonal anti-AtCHL antibody. Bottom panel: Coomassie brillant blue-stained RuBisCO band shown as a load control.

CTRL, control non-treated plant; NaCl, high salt treatment; paraquat, oxidative stress treatment; DH, dehydration treatment; HS, heat shock; LT, low temperature; HL, high light; ABA, abscisic acid.

Figure 3. Modulation of the AtCHL protein level and phenotypic analysis.
(a) Genomic organization of the Arabidopsis SALK_133049 line carrying a T-DNA insertion in the AtCHL gene (At3g47860). Boxes 1–5 are exons.
(b) AtCHL protein levels in leaf extracts. Top panel: immunoblot analysis using the rabbit polyclonal anti-AtCHL antibody. Bottom panel: Coomassie brilliant blue-stained RuBisCO band shown as a load control.
(c) Plants were grown under normal temperature and photoperiod conditions for 21, 28 and 35 days.
(d) Photosynthetic electron transport efficiency (ΔF/ΔFm) measured in attached Arabidopsis leaves (WT, CHL KO, CHL OEX) at various PFDs. Data are mean values of three separate measurements ± SD.
WT, wild-type Col-0 plants; KO, AtCHL knockout line from the SALK collection; Comp, KO plant complemented by over-expression of AtCHL; OEX, an AtCHL over-expressing line; vector, WT transformed with the empty binary vector pPZP121 (negative control).

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AtCHL protein accumulation. RT-PCR analysis showed that plants from this SALK line do not accumulate AtCHL mRNA (data not shown), confirming that they are knockout plants (KO). Complementation (Comp) and over-expressing (OEX) lines were generated by constitutive expression of AtCHL cDNA in the KO and WT Col-0 backgrounds, respectively. All single-insertion Comp and OEX lines analyzed showed very high AtCHL protein accumulation compared to the WT plants, so only one Comp and one OEX line were characterized further (Figure 3b). When grown under normal conditions, plants from all lines germinate, develop, flower and mature similarly, showing that wide variations in AtCHL protein accumulation do not lead to obvious phenotypic differences in non-stressed plants (Figure 3c).

The chloroplastic localization of AtCHL prompted us to determine its possible involvement in the activity of the photosynthetic apparatus. Using chlorophyll fluorometry, we measured the quantum yield of photosynthetic electron transport in Arabidopsis leaves treated at various photon flux densities (PFDs; Figure 3d). As expected, the quantum yield of all lines decreases with increasing PFD, due to progressive saturation of photosynthesis by light. No significant difference was found between WT, CHL KO and CHL OEX lines (Figure 3d) and the empty vector line (data not shown), indicating that removing or over-expressing AtCHL does not affect the photosynthetic performance of leaves. Similarly, HPLC analyses of photosynthetic pigments (chlorophylls and carotenoids) and α-tocopherol did not reveal any differences between WT, KO and OEX plants (data not shown). We also analyzed the fatty acid composition of leaves under control conditions, and did not find any significant difference between WT and the AtCHL mutants (Table S1).

Our previous studies indicated that the plasma membrane-associated lipocalin Atil increases tolerance to freezing and oxidative stresses (Charron et al., 2008). In order to determine whether AtCHL is also involved in abiotic stress tolerance, we assessed the effect of freezing, dehydration and oxidative stresses on the various lines. All lines show similar freezing tolerance (data not shown), providing evidence that AtCHL is not a major player in freezing tolerance and that the TILs and CHLs do not share all the same functions. In the case of dehydration (7 days of water deprivation), AtCHL KO plants do not show significant differences in terms of damage compared to the WT plants, as revealed by the number and size of necrotic lesions on leaves (Figure 4a). In contrast, Comp and OEX lines are much healthier, indicating that the high accumulation of AtCHL increases dehydration tolerance. To quantify stress tolerance, the survival rate was assessed after a 3-day recovery period following dehydration. A lower survival rate was observed in the KO plants compared to WT; however, the difference was not statistically significant (Figure 4b). In contrast, OEX plants showed a significantly higher survival rate compared to the WT and KO plants.

One of the consequences of drought stress is oxidative stress. By inducing closure of the stomata, drought stress reduces the CO₂ supply to the chloroplasts, leading to excess light absorption relative to energy utilization by the photosynthetic processes. To directly generate an oxidative stress, plants were treated with paraquat, an herbicide that generates toxic superoxide radicals at the level of photosystem I. KO plants showed more necrotic lesions and were markedly more affected than WT plants, whereas OEX plants were significantly more resistant (Figure 4c). Quantitative measurement of the survival rate 10 days after paraquat treatment, expressed as a percentage of plants, for 3 weeks, watered thoroughly then allowed to drain (0 day). After 7 days of water deprivation (+7 day), they were rehydrated for a 3-day recovery period (recov.). (b) Survival rate of plants after 3 days of recovery following 7 days of dehydration, expressed as a percentage of plants. (c) Plants were grown under normal conditions for 3 weeks and then sprayed until run off with a 15 μM paraquat solution. The photographs were taken at the indicated time after treatment. (d) Survival rate of plants 10 days after paraquat treatment, expressed as a percentage of plants. WT, wild-type Col-0 plants; KO, AtCHL knockout line from the SALK collection; Comp, KO plant complemented by over-expression of AtCHL; OEX, an AtCHL over-expressing line; vector, WT transformed with the empty binary vector pPZP121 (negative control).
AtCHL alleviates membrane lipid peroxidation

Our goal was to understand how AtCHL enhances tolerance to oxidative stress. Based on the localization of AtCHL in the thylakoid, the inverse relationship between stress sensitivity and AtCHL protein levels, and on the mode of action of paraquat, we hypothesized that this protein might be involved in the protection of chloroplastic membranes against the detrimental effects of ROS. We therefore measured lipid peroxidation in plants of the various lines. In preliminary experiments, we first determined that the level of malondialdehyde, an end product of lipid peroxidation, is similar in all lines grown under normal conditions (Figure S1a). In contrast, when subjected to dehydration or paraquat stress, KO plants show a higher level of peroxidated lipids, whereas the Comp and OEX lines have lower levels (Figure S1b,c). These data support our hypothesis that the AtCHL protein prevents lipid peroxidation. In the case of dehydration, this effect is not mediated via ABA as exogenous ABA treatment alone did not influence the level of peroxidated lipids (Figure S1d).

To assess the effect of AtCHL on lipid peroxidation more accurately, in planta imaging and HPLC analyses were performed. Whole plants aged 5 weeks were exposed to high light stress at low temperature for 24 h. The combination of high light and low temperature is particularly suitable to induce oxidative stress in chloroplasts. During cold treatment, the activity of enzymes of the Calvin cycle is reduced, therefore the incoming light energy funneled into the electron transport chain becomes excessive. This can eventually cause over-excitation of the photosystems and over-reduction of the electron carriers, leading to excitation/electron ‘leakage’ to molecular oxygen and production of ROS. This treatment caused bleaching of most of the mature leaves of KO plants, while OEX plants did not show any symptoms (Figure 5a, left panel). In the WT and empty vector plants, only the tip of some leaves were bleached. Oxidative stress was also visualized in whole plants using autoluminescence imaging. This technique measures the faint light emitted by triplet carbonyls and $^{1}$O$_2$, by-products of the slow spontaneous decomposition of lipid hydroperoxides and endoperoxides (Havaux et al., 2006). This technique has been previously used to map lipid peroxidation and oxidative stress in various biological materials including detached leaves (Flor-Henry et al., 2004), whole plants (Johnson et al., 2007; Collin et al., 2008), animals (Kobayashi et al., 1999) and humans (van Wijk et al., 2006). As shown in Figure 5a (middle panel), leaf autoluminescence is much higher in AtCHL KO plants relative to WT, empty vector and AtCHL OEX plants, indicating that lipid peroxidation is enhanced when AtCHL is absent from the chloroplasts. For comparison purposes, we also exposed Arabidopsis plants deficient in the plasma membrane-associated lipocalin AtTIL (TIL KO) and plants over-expressing AtTIL (TIL OEX) (Charron et al., 2008). In contrast with AtCHL KO plants, light stress did not induce extensive leaf bleaching or lipid peroxidation in TIL KO plants, indicating that AtTIL is not as necessary as AtCHL for preventing oxidative stress generated by light in the chloroplasts. The photosensitativity of AtCHL KO plants was confirmed by thermoluminescence analyses of lipid peroxidation in leaves exposed to high light stress for 1 day (Figure 5b). Thermal cleavage of lipid hydroperoxides, leading to the production of triplet carboxyls, is associated with photon emission at ca. 130°C (Vavilin and Ducruet, 1998; Havaux, 2003). Accordingly, the amplification of lipid hydroperoxides was confirmed by thermoluminescence analysis (Figure 5b).
magnitude of the thermoluminescence band peaking at this temperature range is correlated with the extent of lipid peroxidation as determined by biochemical measurements (Vavilin and Ducruet, 1998; Muller-Moulé et al., 2003; Johnson et al., 2007). The amplitude of the 135°C thermoluminescence band of AtCHL KO plants was much higher than that of signals measured in the other genotypes, indicating accumulation of lipid hydroperoxides in the AtCHL KO plants. Based on the thermoluminescence emission, AtCHL KO plants appear to be the most tolerant to high light stress at low temperature, while WT and the other mutants (TIL KO, TIL OEX, empty vector) are intermediate between AtCHL KO and AtCHL OEX plants.

We used HPLC to quantify the oxidation of linolenic acid (18:3), the major polyunsaturated fatty acid in plant leaves. The concentration of the oxidation product of 18:3, hydroxyoctadecatrienoic acid (HOTE), was calculated as the sum of the various HOTE isomers 9-, 12-, 13- and 16-HOTE. Figure S2 shows typical HPLC traces for WT and AtCHL KO leaves under control conditions (Figure S2a) or after light stress (Figure S2b). 13-HOTE produced enzymatically by lipoxygenase was separated from ROS-produced 13-HOTE using the procedure developed by Montillet et al. (2004). Under normal growth conditions (before stress), the HOTE content was similar (approximately 10 nmol g⁻¹ fresh weight) in WT and the KO and OEX mutants. HOTE levels remained very low throughout the light stress experiment in OEX plants (Figure 6a). In contrast, the HOTE level rose rapidly in KO leaves, peaking at approximately 100 nmol g⁻¹ after a 24 h exposure to high light stress. WT was intermediate between KO and OEX, with the HOTE concentration increasing slightly and monotonously during the high light treatment. However, when the light treatment was prolonged up to 48 h, the HOTE content of KO leaves decreased back to the WT level. This suggests that some adaptive phenomena were induced in stressed KO leaves that increased their tolerance to high light and stimulated the reduction and/or degradation of lipid hydroperoxides. Similarly to WT, the empty vector and TIL OEX exhibited a slight increase in HOTE when exposed to high light (Figure 6b). In the experiment shown in Figure 6, the HOTE level in the empty vector line appeared to be slightly lower than that in WT. However, the difference was not significant. The HOTE content appeared to increase slightly more in TIL KO, reaching approximately 27 nmol g⁻¹ after 24 h, which represents less than a third of the HOTE accumulation in AtCHL KO. Taken together, the results of the luminescence and HPLC analyses of lipid peroxidation demonstrate that AtCHL-deficient leaves are highly sensitive to increased light intensities, and accumulated oxidized lipids upon exposure to high light. The absence of TIL appears to have less effect on the lipid hydroperoxide level compared to the AtCHL deletion. In contrast to ROS-induced lipid peroxidation, lipoxygenase-dependent lipid peroxidation was not enhanced in AtCHL KO plants relative to WT (data not shown).

We analyzed the lipid peroxidation signature by determining the distribution of the HOTE isomers using HPLC-MS/MS. 10- and 15-HOTE are specific to 1O₂-induced lipid peroxidation, whereas the 9- and 16-HOTE isomers can be produced by all ROS, i.e. 1O₂ and free radicals (Mueller et al., 2006; Przybyla et al., 2008). Consequently, when non-enzymatic oxidation of linolenic acid is induced by free radicals, the lipid peroxidation signature is characterized by the absence of 10- and 15-HOTE (Triantaphylides et al., 2008). In contrast, 1O₂-mediated lipid peroxidation leads to the accumulation of 10- and 15-HOTE, which can represent up to 40% of the sum of HOTEs (10-, 15-, 9- and 16-HOTEs). Moreover, it has been demonstrated that, in leaf tissues under optimal growth conditions, basal lipid peroxidation is mainly due to 1O₂, with the corresponding HOTE signature (Triantaphylides et al., 2008). This was confirmed in WT and the AtCHL mutants (data not shown). In OEX plants subjected to high light treatment, lipid peroxidation remained low and the distribution of HOTE isomers (Figure 7) remained typical of 1O₂-induced lipid
peroxidation. Figure 7 shows that high light does not change the HOTE isomer proportions in leaves of the various lines. Thus, the enhanced peroxidation of lipids in AtCHL KO measured in Figure 6 may be attributed to 1O2 attack on polyunsaturated lipids. It may be concluded that AtCHL has a function in prevention of 1O2-mediated lipid peroxidation in high light, hence excluding the possibility that the primary function of AtCHL is to modulate the accumulation of superoxide and H2O2 in the chloroplast, which would have led to different HOTE signatures (Triantaphylides et al., 2008).

We also monitored the photochemical efficiency of photosystem II (PSII) (Fv/Fm) during high light stress (Figure 8). Rapid inhibition of the PSII activity was observed, from 0.8 to approximately 0.2 within 5 h in all genotypes. Fv/Fm remained constant in WT and OEX, but a nearly complete inhibition of PSII accompanied photo-oxidative damage in KO plants, with Fv/Fm decreasing to c.<0.05 after 26 h in high light. Thus, the initial stages of light stress during which lipid oxidation products accumulate in KO leaves (see Figure 6) are not associated with a preferential impairment of PSII photochemistry in KO leaves compared to WT or OEX leaves. Therefore, it appears that the primary cause of lipid peroxidation in AtCHL KO Arabidopsis plants does not stem from an increased photosensitivity of the photochemical activity of the AtCHL-depleted chloroplasts.

**DISCUSSION**

We recently showed that the Arabidopsis temperature-induced lipocalin AtTIL extends the vegetative phase and delays aging (Charron et al., 2008), while other recent studies showed that over-expression of the human lipocalin apolipoprotein D extends life span in mice and *Drosophila* (Ganfornina et al., 2008; Muffat et al., 2008). Unlike these other lipocalins, AtCHL over-expression did not lead to any delay in aging under normal growth conditions. In addition, plants that do not accumulate AtCHL showed no phenotypic changes compared to WT, indicating that the protein is not required for normal growth and development. The available evidence thus suggests that the two Arabidopsis lipocalins do not share the same functions during normal development. Their characterization was thereafter focused on the responses to abiotic stresses. We showed that AtTIL increases tolerance to freezing and paraquat (Charron et al., 2008). In the case of AtTIL, clear phenotypic differences are observed when plants are subjected to dehydration or oxidative stress conditions, with the level of AtCHL accumulation being associated with the level of tolerance. Compared to WT plants, KO plants show a higher level of hydroxy fatty acids, which is associated with a decrease in tolerance. Plants that accumulate high levels of the protein show much lower levels of peroxidated membrane lipids and a concomitant marked increase in tolerance to oxidative stress. Up-regulation of the AtCHL gene under various oxidative stress conditions and modulation of the tolerance of Arabidopsis plants as a function of the AtCHL protein level strongly support the notion that this lipocalin represents a defense mechanism against oxidative stress. When Arabidopsis plants are exposed to high light stress at low temperature, absence of AtCHL has a stronger impact than absence of AtTIL, indicating that the former lipocalin is more necessary for tolerance to photo-oxidative stress generated in the chloroplast. Conversely, AtTIL increases the freezing
resistance of Arabidopsis seedlings (Charron et al., 2008) whereas AtCHL does not, confirming that the protective activities of AtTIL and AtCHL are specific to different stress conditions. Moreover, a high level of accumulation of AtCHL in the OEX mutant did not modify the basal level of oxidized lipids under normal growth conditions. Therefore, the protective role of AtCHL seems to be specific to oxidative stress conditions rather than to normal cellular metabolism. In our experiments, the protection provided by AtCHL was mainly visible during the early stages of the stress; prolonged treatments did not lead to significant differences in lipid peroxidation between KO and WT plants. Possibly, AtCHL represents a rapid response to acute oxidative stress conditions. In the long term, other protective mechanisms may come into play, which would compensate for the loss of AtCHL.

The lipids constituting the thylakoid membranes are known to be very unsaturated, and are therefore very sensitive to oxidation. As a consequence, thylakoid membranes contain a variety of lipid-soluble anti-oxidants that protect them against lipid peroxidation, including carotenoids, tocopherols, plastoquinone and volatile terpenoids (Triantaphylides and Havaux, 2009). Among the carotenoids, zeaxanthin appears to have a central role as lipid protector (Johnson et al., 2007). Chloroplasts also contain enzymatic systems that can detoxify lipid hydroperoxides, such as peroxiredoxins (Manevich et al., 2008). In Drosophila, over-expression of human apolipoprotein D was shown to lower the level of lipid peroxides (Muffat et al., 2008). In contrast with the Arabidopsis TIL, AtCHL is not a membrane-bound protein, as it was detected exclusively in the thylakoid lumen. AtTIL has been found to be attached to the plasma membrane (Charron et al., 2008) and to physically interact with plasma membrane lipids (Tominaga et al., 2006). Interestingly, the wheat chloroplastic lipocalin TaCHL has been reported to interact with the LTP3 lipid transfer protein 3 (Tardif et al., 2007). Members of the LTP family are known for their ability to mediate the in vitro transfer of phospholipids between membranes and for their role in plant defense (Boutrot et al., 2008). The question arises as to how AtCHL, as a soluble protein, can participate in modulation of the level of membrane lipid peroxidation in chloroplasts. AtCHL knock-out or over-expression did not affect the sensitivity of photosystem II to photo-inhibition. Indeed, during the early stages of light stress, the accumulation of hydroxy fatty acids in the AtCHL-deficient mutant was not associated with enhanced photo-inhibition, suggesting that the lipid protective function of the protein did not occur primarily through stabilization of the photochemical apparatus of photosynthesis. Consequently, a more direct action of AtCHL must be considered with regard to the sensitivity of membrane lipids to oxidation.

In animals, lipocalins have been shown to bind a variety of lipid ligands, including lipid oxidation products (Grzyb et al., 2006). In vivo binding of human tear lipocalin 1 (Lcn-1) to F2 isoprostanes, which are typical lipid peroxidation products of arachidonic acid, has been reported (Lechner et al., 2001). The biological function of Lcn-1 is believed to be scavenging of potentially harmful lipophilic molecules from the mucous surface of the eye to the liquid phase (Gasymov et al., 2005). We speculate that AtCHL could play a similar scavenging role in plant chloroplasts. Plants do not synthesize arachidonic acid and thus do not form isoprostanes. Instead, they utilize linolenic acid to synthesize phytoprostanes via a ROS-catalyzed pathway analogous to the isoprostane pathway in animals (Thoma et al., 2004). As in animals, polyunsaturated fatty acid hydroperoxides and peroxy radicals in plant lipid membranes undergo fragmentation to produce various reactive intermediates, called reactive electrophile species. One such compound is malondialdehyde, which was found to accumulate to higher levels in AtCHL KO plants compared with WT and OEX plants in response to various oxidative stresses. Reactive electrophiles are harmful to macromolecules by reacting with nucleophilic groups, resulting in a variety of adducts and irreversible modifications. Compared to ROS, reactive electrophile species are stable, and, due to their non-charged structure, a number of these reactive compounds can migrate through hydrophobic membranes and hydrophilic media. They are thus able to propagate oxidative stress far from their site of formation (Pamplona, 2008). Scavenging of these lipid peroxidation intermediates in the chloroplast is potentially an important mechanism to avoid propagation of oxidative damage to other thylakoid constituents or other cellular compartments, and AtCHL appears to be a possible candidate for such a function. It is clear that, in future work, binding analyses should be performed on AtCHL to address this hypothesis and determine the precise mode of action of AtCHL.

In addition to a clearance function, AtCHL could also reinforce the tolerance of thylakoid membranes to oxidative damage by transporting and delivering lipophilic anti-oxidants. As mentioned above, the two main anti-oxidants in the thylakoid membranes are the carotenoids and tocopherols. In the human eye, α-tocopherol is present in the lipid phase of tears, and half of the tocopherol pool is bound to protein, a large part of which can be recovered from purified fraction of tear lipocalin (Glasgow et al., 2002). On the other hand, some animal lipocalins or plant lipocalin-like proteins, such as crustacyanin, violaxanthin de-epoxidase and zeaxanthin epoxidase, are known to bind xanthophyll
carotenoids (Gryzb et al., 2006). Although we cannot exclude the possibility of a similar interaction between tocopherols or carotenoids and AtCHL, several observations argue against this hypothesis. First, the last steps of tocopherol synthesis occur in the plastoglobules, which are physically connected to thylakoids (Brehelin and Kessler, 2008), so tocopherol delivery from the lumen seems unlikely. Second, carotenoid synthesis occurs in the thylakoids and the chloroplastic envelope (Cunningham and Gantt, 1998), implying carotenoid trafficking between these membrane compartments through the stroma, not through the lumen. Lastly, AtCHL over-expression or deletion in Arabidopsis has no impact on tocopherol and carotenoid content. A role in lipid transport for membrane biogenesis or maintenance also seems unlikely as the fatty acid content and composition of the leaves is independent of the level of AtCHL. Moreover, fatty acid biosynthesis involves complex trafficking between the stroma, the cytosol, the chloroplast envelope and the thylakoids, but it does not involve the thylakoidal lumen.

To sum up, this study has shown that chloroplastic lipocalin AtCHL is involved in the anti-oxidant defense of Arabidopsis leaves against light-induced oxidative stress. Mutational suppression of CHL results in a fast accumulation of hydroxy fatty acids under conditions of excess light energy, while constitutive over-accumulation of the protein maintains lipid peroxidation at a very low level, and these effects are correlated with tolerance to photo-oxidative damage. We conclude that this protein has an essential role in the response of Arabidopsis to stress, by managing or preventing lipid peroxidation.

**EXPERIMENTAL PROCEDURES**

**Plant growth conditions and treatments**

Arabidopsis ecotype Columbia (Col-0) was the genetic background of all lines used. The SALK_130949 line (KO) has a T-DNA insertion in the AtCHL gene (At3g47860), and was obtained from the Arabidopsis Biological Resource Center (Ohio State University). Seeds were sown on agar plates containing 50 μg ml⁻¹ kanamycin, stratified for 2 days at 4°C, and grown at 22°C. Kanamycin-resistant plants were propagated as individual lines on potting medium, watered thoroughly, allowed to drain for 1 day and water-deprived for 7 days. They were then rehydrated for 3 days (recovery period). For heat shock, plants were treated at 40°C for 1 h. For low temperature, plants were treated at 4°C for 24 h. For the high light treatment, plants were exposed to light at a PFD of 1000 μmol m⁻² sec⁻¹ for 24 h. For short stress treatments (3 and 6 h), plants were grown on plates and treated with 15 μm paraquat or dehydrated by removing the plants from the agar. For ABA treatment, plants were treated with a 100 μm solution of (-)-abscisic acid (Sigma-Aldrich).

For the pigment and lipid peroxidation analyses, plants were grown for 5 weeks in a phytotron at 16°C, then fractionated into their sub-organellar constituents. The chloroplasts contained thylakoids. The proteins in the supernatant were concentrated by trichloroacetic acid (TCA) precipitation. The thylakoids contained the soluble stromal and envelope proteins and the pellet contained thylakoids. The proteins in the supernatant were concentrated by trichloroacetic acid (TCA) precipitation. The thylakoids were washed briefly three times with 10 mM Tris/HCl pH 8.0, and resuspended in 10 mM Tris/HCl pH 8.0, 5 mM MgCl₂ at a chlorophyll concentration of 0.5 mg ml⁻¹, then sonicated 10 times for 30 sec each at 4°C (Sonic Dismembrator Model 500, Fisher Scientific). Samples were ultracentrifuged for 1 h at 145 000 g at 4°C to recover the thylakoid membranes in the pellet and the soluble luminal proteins in the supernatant. The latter were concentrated by TCA precipitation. All samples were subjected to SDS-PAGE and immunoblot analyses.

**Isolation and fractionation of intact chloroplasts**

Intact chloroplasts were isolated and purified on Percoll gradients (Cline, 1986), then fractionated into their sub-organelar constituents (Peltier et al., 2000). All solutions contained Complete™ protease inhibitor cocktail (Roche, http://www.roche.com). Chloroplasts (30 mg chlorophyll) were ruptured by osmotic shock in 30 ml of 50 mM Tris/HCl pH 8.0, 5 mM MgCl₂ for 10 min at 4°C. Following centrifugation at 10 000 g for 10 min at 4°C, the supernatant contained the soluble stromal and envelope proteins and the pellet contained thylakoids. The proteins in the supernatant were concentrated by trichloroacetic acid (TCA) precipitation. The thylakoids were washed briefly three times with 10 mM Tris/HCl pH 8.0, and resuspended in 10 mM Tris/HCl pH 8.0, 5 mM MgCl₂ at a chlorophyll concentration of 0.5 mg ml⁻¹, then sonicated 10 times for 30 sec each at 4°C (Sonic Dismembrator Model 500, Fisher Scientific, http://www.thermofisher.com). Samples were ultracentrifuged for 1 h at 145 000 g at 4°C to recover the thylakoid membranes in the pellet and the soluble luminal proteins in the supernatant. The latter were concentrated by TCA precipitation. All samples were subjected to SDS-PAGE and immunoblot analyses.

**Immunoblot and RT-PCR analyses**

The AtCHL cDNA was cloned into the pTrc-His vector (Invitrogen, http://www.invitrogen.com/) and electroporated into Escherichia coli strain DH5α. The recombinant His₆-AtCHL protein was produced by induction with 1 mM IPTG for 3 h. The cells were collected, broken by lysozyme treatment and sonication, and His-tagged...
proteins were purified by immobilized metal affinity chromatography on Ni-NTA agarose (Novagen, http://www.emdbiosciences.com). The purified proteins were over 90% pure and were used to immunize a rabbit to obtain anti-AtCHL polyclonal antibodies.

Aerial parts of Arabidopsis plants were cut and immediately frozen in liquid nitrogen. Proteins and total RNA were isolated concomitantly from leaves (100 mg) using TRIzol® according to the manufacturer’s instructions (Invitrogen). For immunoblot analyses, protein samples were separated on 12% SDS-PAGE gels, and detection was performed using the rabbit anti-AtCHL antibody (1:10 000), a peroxidase-coupled anti-rabbit IgG secondary antibody (1:25 000), and Western Lightning Chemiluminescence Reagent Plus (PerkinElmer Life Sciences). For RT-PCR, total RNA was subjected to reverse transcription using SuperScript™ III reverse transcriptase (Invitrogen) according to the manufacturer’s recommendations. Primers were designed to specifically amplify the Arabidopsis AtCHL (AtCHL-F, 5′-TGAAAGTAGCTCCTCTTAAGCG-TGG-3′; AtCHL-R, 5′-ATATATCCATCAAGGCCGTCA-GA-3′) and actin control transcripts (Act-F, 5′-TCAGATGCCCCAGAAGTGTGT-3′; Act-R, 5′-CCGTACAGATCTCCTGATA-3′). PCR products were analyzed by electrophoresis on 1% agarose/ethidium bromide gels.

Lipid peroxidation analyses

Preliminary determination of lipid peroxidation levels in the various lines was performed using the thiobarbituric acid test, which determines the amounts of malondialdehyde as the end product of lipid peroxidation (Heath and Packer, 1968; Loreto and Velikova, 2001; Sunkar et al., 2003). For a more accurate and quantitative determination, peroxidized lipids were extracted and analyzed by HPLC. Lipids were extracted from approximately 0.5 g frozen leaves by grinding with 2 × 1 ml CHCl3 containing 1 mg ml−1 triphenyl phosphate and 0.05% v/v butylated hydroxytoluene, with 15-hydroxy-eicosatrienoic acid (HOTE) isomers as previously described using 15-hydroxy-eicosatrienoic acid as an internal standard. The organic phase was evaporated under a stream of N2. The residue was recovered in 1.25 ml ethanol and 1.25 ml 3.5 mM NaOH, and hydrolyzed at 80°C for 15 min. After addition of 2.2 ml 1 M citric acid, hydroxy fatty acids were extracted with 2 × 1 ml hexane/ether (50/50). An aliquot of the organic phase (50 μl) was submitted to straight-phase HPLC (Waters, http://www.waters.com) using a Zorbax Rx-SIL column (4.6 × 250 mm, 5 μm particle size, Agilent, http://www.agilent.com), isocratic elution with 70/30/0.25 v/v/v hexane/diethyl ether/acetic acid at a flow rate of 1.5 ml min−1, and UV detection at 234 nm. ROS-induced lipid peroxidation was evaluated based on the levels of the various hydroxyoctadecatrienoic acid (HOTE) isomers as previously described using 15-hydroxy-eicosatrienoic acid as the internal standard (Montillet et al., 2004). Lipoxigenase (LOX)-induced lipid peroxidation was estimated from the level of 13-HOTE after subtraction of racemic 13-HOTE (attributable to ROS-mediated lipid peroxidation) (Montillet et al., 2004).

The distribution of hydroxy fatty acid isomers was analyzed by electrophoresis on 1% agarose/ethidium bromide gels.

Thermoluminescence and autoluminescence imaging

Lipid peroxidation was also measured directly in leaf discs by thermoluminescence using a custom-made apparatus that has been described previously (Havaux, 2003). The amplitude of the thermoluminescence band peaking at approximately 135°C was used as an index of lipid peroxidation (Ducruet, 2003; Havaux, 2003).

The distribution of hydroxy fatty acid isomers was analyzed by electrophoresis on 1% agarose/ethidium bromide gels.


