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Inter-kingdom complementation discloses functional convergence of lipocalins.

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Abstract

Background

The chloroplastic lipocalin (LCNP) is induced in response to various abiotic stresses including high light, dehydration and low temperature. It contributes to protection against oxidative damage promoted by harmful conditions by preventing accumulation of hydroxy fatty acids and lipid peroxidation. In contrast to animal lipocalins, LCNP is poorly characterized and the molecular mechanism by which it exerts protective effects during oxidative stress is largely unknown. LCNP is considered the ortholog of human apolipoprotein D (APOD), a protein whose lipid antioxidant function has been characterized. Here, we investigated whether APOD could functionally replace LCNP in Arabidopsis.

Results
We introduced APOD cDNA fused to a chloroplast transit peptide encoding sequence in an Arabidopsis LCNP KO (knock-out) mutant line and challenged the transgenic plants with different abiotic stresses. We demonstrated that expression of human APOD in Arabidopsis can partially compensate for the lack of the plastid lipocalin. However, if the results obtained with the drought and oxidative stresses point to the protective effect of constitutive expression of APOD in plants lacking LCNP, this effect is not as effective as that conferred by LCNP overexpression. Moreover, when investigating APOD function in thylakoids after high light stress at low temperature, it appeared that APOD could not contribute to qH, a slowly reversible form of non-photochemical chlorophyll fluorescence quenching, as described for LCNP.

Conclusions

The results are consistent with a conserved function of APOD and LCNP under stressful conditions. The work contributes towards understanding the molecular mechanism of action of LCNP in oxidative stress protection and provides the basis for new research assumptions.

Keywords: Apolipoprotein D, Lipocalin, Chloroplast, Oxidative stress, Arabidopsis
Background.

Lipocalins constitute an evolutionary conserved family of small proteins widely distributed in nature whose common feature is their ability to bind small hydrophobic molecules [1]. Although their primary amino acid sequences are not highly conserved, they all display a repeated structurally conserved region and an eight-stranded antiparallel β-sheet that together form a compact barrel structure with a calyx-shaped ligand pocket [2]. Shape, size and properties of this pocket vary among family members in line with the different ligands that can be accommodated. A large variety of ligands including lipids, steroids as well as secondary metabolites such as vitamins, cofactors and odorants have been reported [1, 3]. Related to their ligands, lipocalins can fulfill a wide variety of functions such as transport of small molecules, regulation of developmental processes, signal transduction and response to stress. Because of their possible involvement in various diseases including lipid disorders, neurodegenerative diseases and cancer [4, 5], human and animal lipocalins are extensively studied.

In striking contrast, plant lipocalins are poorly characterized. The first lipocalins reported in plants were the violaxanthin-de-epoxidase (VDE) and the zeaxanthin epoxidase (ZEP), two enzymes involved in the xanthophyll cycle [6]. Given the peculiar molecular and structural features of both enzymes and the strict definition of lipocalins, VDE and ZEP are now classified as lipocalin-like proteins [7]. Genomic data mining demonstrated that plants possess also true lipocalins, which were classified as temperature-induced lipocalin (TIL) and chloroplastic lipocalin (LCNP) [7]. Investigation of gene regulation, proteomic analyses and reverse genetic studies have related TIL and LCNP with stress response and tolerance. TIL appeared essential for thermotolerance [8], its accumulation during cold acclimation was described in Arabidopsis leaves [9] and in Siberian spruce needles [10]. TIL gene expression has been demonstrated to

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1 Initially named CHL, the chloroplast lipocalin was renamed LCNP [“Lipocalin in the Plastid”] by Malnoë et al. in 2018. This abbreviation will be used throughout the text for designating the plastid lipocalin.
increase with heat and cold treatment in wheat and Arabidopsis [11] and peach fruits [12]. In *Oryza sativa*, a TIL encoding gene was reported to be highly up regulated under heat stress in panicles of tolerant rice cultivar [13]. TIL expression was also significantly induced by a cold treatment in *Medicago sativa* subsp. *falcata*, a forage legume cold tolerant [14] and in a grapefruit following a conditioning treatment that enhances chilling tolerance [15]. In grape berries of *Vitis labruscana*, salicylic acid treatment up-regulated TIL preventing postharvest loss during cold storage [16]. In mango fruits a TIL homolog and its corresponding gene appeared to be up-regulated during a brassinolide-mediated response to cold stress [17]. Moreover, TIL plays a role in salt tolerance in *Populus euphratica*, a salt-tolerant poplar species [18, 19].

Considering LCNP, it has been demonstrated that high light, dehydration, oxidative stress and abscisic acid (ABA) induced transcript and protein accumulation in Arabidopsis [20]. In wheat, cold acclimation induced LCNP mainly in freezing-tolerant cultivar while heat exposure down-regulated gene expression [7]. In *Festuca arundinacea*, salt stress induced a LCNP protein level increase, which was higher in a salt-tolerant genotype [21]. Furthermore, LCNP abundance similarly decreased in response to drought treatment in two cultivars of *F arundinacea*, however, after rewatering lipocalin level returned to initial level only in the high-drought-tolerant genotype [22].

In spite of the accumulating knowledge regarding the contribution of TIL and LCNP to the resistance of plants to environmental constraints little is known about their role at the cellular and molecular levels. It has been suggested that plant lipocalins prevent accumulation of hydroxy fatty acids and thus stop the lipid peroxidation chain reactions initiated by reactive oxygen species (ROS) generated during stresses [8, 23]. In 2009, Levesque-Tremblay et al. [20] demonstrated that when exposed to excess light, LCNP KO plants display a rapid accumulation of hydroxy fatty acids relative to the wild-type, whereas the lipid peroxidation level remains
very low in LCNP overexpressing plants. Boca et al. [24] demonstrated that LCNP deficiency dramatically enhances the photosensitivity of mutants affected in lipid protection mechanisms strengthening the hypothesis that lipocalins prevent lipid peroxidation. Interestingly, recent data obtained by Malnoë et al. [25] demonstrated a link between LCNP and qH a slowly reversible form of non-photochemical energy quenching (NPQ), a mechanism by which photosynthetic organisms harmlessly dissipate excess absorbed light energy. The authors suggested that the accumulation of peroxidized lipids observed in Arabidopsis lcnp mutants following abiotic stress is a consequence of the absence of the photoprotective NPQ mechanism enabled by the plastid lipocalin and, thus, that LCNP might function in preventing the formation of peroxidized lipids. In addition to or as part of its role in NPQ, it is suggested that LCNP might detoxify peroxidized lipids directly [25].

Phylogenetic studies established that plant lipocalins share similarities with the bacterial lipocalin, the mammalian apolipoprotein D (APOD) and the insect Lazarillo protein. As observed in plants, the correlation between lipid oxidation status and lipocalin abundance was reported for animal and human organisms. For instance in mice, loss of APOD increased lipid peroxidation in the brain [26]. Conversely, human APOD overexpression reduced lipid peroxidation in mice and Drosophila [26, 27]. Increased APOD levels in the human brain were reported under conditions that promote lipid peroxidation such as aging or Alzheimer’s disease [28]. At the biochemical level, APOD ligands are well-known [29, 30, 31] and the molecular mechanism underlying the antioxidative properties of APOD has been established. Indeed, the ability for APOD to control lipid peroxidation by catalyzing the reduction of radical-propagating lipid hydroperoxides has been characterized [32].

In order to characterize the mechanism underlying the protective role of LCNP against abiotic stresses we investigated whether APOD whose biochemical properties involved in oxidative stress tolerance have been described, is functionally orthologous to the chloroplast lipocalin.
To do so, human APOD cDNA fused to a chloroplast transit peptide encoding sequence was introduced in an Arabidopsis LCNP KO mutant line. Transgenic plants expressing APOD were challenged with different abiotic stresses. The plants displayed partial tolerant phenotype suggesting that human APOD can replace in part, LCNP. These data provide a base of understanding the antioxidative mechanism of the plastid lipocalin and some testable experimental assumptions.

Results

Targeting the human APOD to Arabidopsis thaliana chloroplast: gene expression and protein localization.

To determine whether APOD is functionally orthologous to LCNP we took an in vivo complementary approach expressing the human APOD encoding sequence in Arabidopsis. In order to target APOD to the chloroplast where LCNP is located, the putative transit peptide encoding sequence of LCNP was fused to the cDNA coding for APOD (Fig. 1A). The chimeric construct under the control of the Cauliflower Mosaic virus (CaMV) 35S promoter was introduced in an Arabidopsis mutant line lacking LCNP [20]. Primary transgenic Arabidopsis plants were identified following Basta treatment of one-week old seedlings. In the T2 generation, 5 independent lines containing a single insertion were selected by analyzing the segregation ratio of herbicide resistance. Seeds were collected from homozygous T3 plants and used for further experiments.

The 5 transformant lines a661, a351, a421, a551, and a212 showed no phenotypic changes compared to wild type. In the five transgenic lines, APOD expression was analyzed by reverse transcriptase polymerase chain reaction (RT-PCR, Fig. 1B). Two lines a661 and a421 exhibited a higher expression as compared to the three other lines. At the protein level, immunoblot
analysis was conducted to detect the presence of APOD (Fig. 1C). A strong band was observed in the two transgenic lines containing the higher amount of APOD transcripts (a661 and a221) while the protein was hardly detectable in the three other lines. The molecular weight of the band was estimated at 23 KDa, which does not correspond to the molecular weight of APOD estimated at 19.3 KDa suggesting either that the transit peptide was not properly processed or that APOD was post-translationally modified.

Within chloroplasts, LCNP was localized in the thylakoid lumen using a cellular fractionation approach [20]. To investigate APOD subcellular localization we performed western-blot experiments on plastid sub-fractions isolated from chloroplasts purified by Percoll gradient centrifugation (Fig. 2). APOD appeared to be of low abundance in the total cell extract. As expected its concentration slightly increased in purified chloroplasts where the human lipocalin was present in both compartments, thylakoids and stroma. However, in NaCl stripped thylakoids APOD could not be detected suggesting that the protein is ionically attached to the membranes. These results suggest that in chloroplasts, APOD remains soluble in the stroma and also ionically attaches to the thylakoids.

To investigate further the localization of APOD in transgenic plants, the protein was tagged with EGFP at its C-terminus and transiently expressed using the CaMV 35S promoter in Nicotiana benthamiana leaves (Fig. 3). Six days later, leaf epidermal cells were observed by confocal microscopy. EGFP fluorescence coincided strongly with chlorophyll autofluorescence (Fig. 3A) indicating that, as expected, APOD:EGFP fusion product was associated with the chloroplast. In particular, confocal imaging revealed a strong fluorescence within the guard cell chloroplasts (Fig. 3A). Furthermore, EGFP fluorescence was also detected in other subcellular structures throughout the cell. To identify EGFP labeled structures co-localization experiments were performed with different compartment markers (Fig. 3B and 3C). Fluorescence was evident in the endoplasmic reticulum network (ER) and in Golgi as demonstrated by the co-
localization of the tagged protein with the mCherry signal targeted either to the ER (Fig. 3B) or to the Golgi (Fig. 3C). In contrast co-localization experiments performed with a marker of plasmalemma and tonoplast excluded the presence of proteins in these membranes (data not shown).

**Effect of APOD expression on abiotic stress tolerance**

The chloroplast lipocalin plays an important role in tolerance to environmental constraints. In Arabidopsis it has been demonstrated that a high level of LCNP helped plants to cope with environmental abiotic stresses such as drought, high light, cold acclimation and oxidative stress. Conversely plants lacking LCNP exhibited more damage under abiotic stresses [20]. In order to investigate whether APOD could also confer stress tolerance and play a role similar to that of LCNP in protecting plants, the transgenic lines (a661 and a421) expressing *APOD* in the *LCNP* KO background were exposed to different stress conditions including drought (Fig. 4) and oxidative stress (Fig. 5).

*Arabidopsis lcnp*, wild-type and transgenic seeds were planted in a mix of soil and sand. Three weeks later, water was withheld for 10 days. *lcnp* mutants showed severe withering symptoms as compared to wild type plants, attesting of the great sensitivity of these plants to a water deficit. The plants over-expressing *LCNP* (*LCNP O/E*) and those expressing *APOD* were only slightly wilted (Fig. 4A). When plants were re-watered, none of the KO mutant plants survived (Fig. 4B). In contrast, *Col0, LCNP O/E* and the two lines containing APOD coped well with the stress conditions. These results suggested that *APOD* expression in the drought-sensitive *lcnp* genotype could restore tolerance to a mild water stress to the level recorded in wild type plants.

Previous studies indicated that LCNP is involved in the protection of the photosynthetic apparatus against ROS. Therefore we have tested resistance of plants against cellular oxidative damage by applying the herbicide paraquat (PQ, methyl viologen) to leaf disks segments from
transgenic plants. The electrons produced during photosynthesis reduce PQ and free oxygen radicals are formed initiating an oxidative stress and quickly damaging the photosynthetic apparatus. Leaf disks were exposed to 1 μM PQ for 8 hours under a light source. Functional damage was monitored by measuring the variable fluorescence (Fv)/maximal fluorescence (Fm) values representing the maximal yield of photochemistry in the photosynthetic process (Fig. 5). The control and lcnp mutant completely lost their photosynthetic function. As expected the LCNP O/E line was significantly more resistant to PQ treatment. Interestingly, plants expressing APOD (a661 and a421) exhibited a moderate resistance while a212, a line exhibiting a very low expression of APOD displayed an oxidative stress-sensitive phenotype similar to the control (Col0). Considering these results we can conclude that the constitutive presence of APOD confers enhanced tolerance to PQ-induced oxidative stress. However oxidative tolerance did not appear as notable as that recorded in LCNP overexpressing plants.

Effect of APOD expression on sustained photoprotective energy dissipation

It has been demonstrated that LCNP contributed to qH, a NPQ component, which operates under stress conditions such as cold and high light and appeared to be photoprotective [25]. Whether APOD can replace LCNP in this function was investigated. Detached leaves of transgenic plants were exposed to high light at low temperature for 6 hours and maximal fluorescence Fm was recorded. The suppressor of quenching 1 mutant (soq1) with enhanced qH whose phenotype is reversed when LCNP is lacking was included in the experiment. Before the stress, Col0 and the different mutant and transgenic lines displayed similar maximal fluorescence parameter (Fm, data not shown). After stress (Fig. 6), as expected since already described by Malnoë et al. [25], soq1 displayed a lower Fm compared with Col0 while Fm in lcnp was higher than in the control plant leaves. The photoprotective function of LCNP was observed in the leaves of the transgenic line overexpressing LCNP with a maximal chlorophyll fluorescence parameter lower than in Col0. In comparison, a661 and a421 displayed a Fm
similar to that recorded in lcnp indicating that APOD expression cannot protect the thylakoid membrane as LCNP does.

Discussion

We report that expression of human APOD in Arabidopsis, by means of transgenic lines, can partially compensate for the lack of the plant lipocalin, LCNP. The results are consistent with a conserved function of APOD and LCNP under stressful conditions. However, if the results obtained with the drought and oxidative stresses point to the protective effect of constitutive expression of APOD in plants lacking LCNP, this effect is not as effective as that conferred by LCNP overexpression. Moreover, when investigating APOD function in NPQ after high light stress at low temperature, it appeared that APOD could not contribute to qH as described for LCNP [25]. It is intriguing to notice that in Arabidopsis, APOD can fulfill only certain functions of LCNP.

Molecular phylogenetic analysis of lipocalins not only revealed that the chloroplastic LCNP shared significant structural homology with the mammalian APOD [7, 33], but also suggested that both proteins might correspond to the same overall function in terms of general nature of binding ligands since both proteins appeared in the same clade [7, 34]. In vitro ligand binding assays have shown that APOD is able to bind a variety of lipids in its hydrophobic binding cavity [29]. More recently, it has been clearly demonstrated that APOD displayed the highest binding affinity for arachidonic acid [AA] and progesterone [29, 30, 31]. Partial functional complementation of LCNP deficiency by APOD cannot be related to the nature of APOD ligands since neither AA nor progesterone are present in Arabidopsis. However we cannot exclude the possible affinity of APOD to other structurally related lipids that may be involved in signaling cascades. Indeed numerous signaling lipids whose synthesis can rapidly be activated upon abiotic stress signals have been identified in plants [for a review see 36].
Arabidopsis, APOD could exert a control of the signaling lipid molecules that decipher the stress response cascade and thus modulate transgenic plants tolerance. In plants, a similar function for LCNP cannot be excluded as well. During last decade, several authors have established that APOD function extended well beyond the transport of lipophilic molecules. Therefore, an extensive work allowed to establish a correlation between lipid oxidation status and APOD abundance in human and mammals. Increased APOD expression was reported in the human brain under conditions that promote lipid peroxidation and up-regulation of antioxidant gene expression such as aging or neuropathological conditions including schizophrenia, Parkinson’s and Alzheimer’s diseases [28, 35, 37]. Studies in mice showed that loss of APOD increased lipid peroxidation in the brain, that cerebral APOD expression is increased in response to Paraquat-induced oxidative stress and that human APOD expression reduced lipid peroxidation [26, 27]. In addition, expression of human APOD reduced the accumulation of aldehydic end-products of lipid peroxidation in an aged Drosophila model [27, 38]. In plants, the protective role of LCNP in oxidative stress has been related to its ability to prevent or modulate lipid peroxidation caused by ROS action [20]. Thus, a LCNP KO Arabidopsis line, which exhibited serious damage upon photo-oxidative stress induced by drought, high light or paraquat displayed a rapid accumulation of hydroperoxyl fatty acids. In contrast, in LCNP over-expressing lines, which coped better with these conditions, the lipid peroxidation level remains very low [20]. Related to APOD antioxidative function, it has been demonstrated that the human lipocalin had the capacities to scavenge hydroxyl radicals [40] and catalyze the reduction of hydroperoxide of AA to the non-reactive alcohols, thereby preventing lipid peroxidation chain reactions [32]. This reaction involves a highly conserved methionine residue (Met93 in human APOD) located at the hydrophobic surface of the protein [32] and results in oxidation of the methionine sulfhydryl to form methionine sulfoxide (MetSO). A similar molecular function could be
involved to explain the protective role of APOD in Arabidopsis. Indeed, it can be speculated that APOD could exert its reductive activity on plant lipid hydroperoxides such as hydroperoxides of linolenic (18:3), linoleic (18:2) and roughanic (16:3) acids, the major polyunsaturated fatty acids in plants. In this context, APOD could inhibit lipid peroxidation propagation initiated by drought and paraquat treatment. A lower affinity of APOD for plant hydroperoxides or an insufficient amount of APOD in plant cells could account for the lower protective effect of the human lipocalin as compared to that provided by LCNP. By analogy, the protective function towards oxidative stresses conferred by LCNP could be due to a surface methionine residue, similar to APOD Met93 and able to reduce plant fatty acid hydroperoxides, as well. In that respect, we investigated whether LCNP could exhibit a methionine residue structurally homolog of APOD Met93. In the absence of an experimentally-determined protein structure, homology modeling using Phyre2 provided a three-dimensional structure for LCNP. Structural alignment with the human APOD model is shown in Fig 7. The APOD Met93 is not conserved in LCNP ruling out the contribution of the Met93 homolog in LCNP oxidative protective function. However, LCNP contains many other Met residues whose putative reductive activity should be investigated in future studies [7].

The appropriate subcellular localization and translocation of lipocalins are crucial to their functionality. LCNP located into the lumen of thylakoids [20] has been shown to be involved in qH, which has been localized to the peripheral antenna of photosystem II, suggesting that LCNP could either directly form a NPQ site or indirectly modify the light harvesting complex II (LHClI) membrane environment [25]. Lumen targeting is a complex process, which may involve two different pathways able to recognize a thylakoid-targeting signal peptide [41]. The N-terminal bipartite signal for chloroplast import and thylakoid transfer that drives the import of LCNP from the cytosol to the lumen has not yet been characterized. In our construct, LCNP transit peptide extension was defined based on unpublished data [Malnoë, personal
In Arabidopsis transgenic plants, APOD fused to the putative transit peptide of LCNP was clearly targeted to the chloroplast (Fig. 2 and Fig. 3). However we could not find evidence that APOD penetrated into the lumen of thylakoids. Inappropriate processing could explain the APOD unexpected size described in Fig. 1 and the lack of APOD in the lumen.

APOD is a glycoprotein and we cannot exclude that glycosylation occurred in cells of transgenic plants. The localization of APOD fused to GFP into Golgi and ER supports this hypothesis (Fig. 3). Moreover, this post-translational modification could explain the unexpected molecular weight of APOD in a plant cell extract (Fig. 2). Lastly, targeting of glycoproteins into chloroplasts is well known [42] but import into thylakoids lumen has never been described. The lack of lipocalin into the chloroplast sub compartment, although the protein is present in the stroma, may explain why APOD did not contribute to qH as LCNP does.

Conclusions

Our data indicate that despite structural homology and similar contribution to lipid oxidation status in cells, APOD and LCNP display molecular features that confer specific functions to them. We propose that functionality and specificity likely reside in a particular methionine residue and glycosylation modifications that affect enzyme activity and subcellular localization, respectively. The findings of this study provide new leads for future research. In particular, we are examining LCNP methionine residues to assess their contribution in lipid hydroperoxide reduction.

Methods

**Plant Material, Growth Conditions and Treatments**

Arabidopsis plants were grown in phytotrons with an 8/16 day/night photoperiod and a light intensity of 130 μmol m\(^{-2}\) s\(^{-1}\). Day and night temperatures were 22 and 18 °C, respectively. The
Relative humidity was set at 60% and plants were cultivated at 100% relative soil water content. For drought stress, 9 plants of each line were grown in a single pot containing 120 g of a mix of soil and sand (2/1, w/w). Three week old plants were then subjected to water deprivation by withholding watering for 10 days. For the oxidative stress treatment, about 9 mm diameter leaf disks were excised from fully expanded leaf blades and floated onto a 1 µM solution of paraquat (PQ, N,N’-dimethyl-4-4’-bipyridinium dichloride, Sigma-Aldrich) dissolved in water for 8 hours.

*Nicotiana benthamiana* plants were grown in soil under controlled conditions in a growth chamber (14-h photoperiod at 25°C followed by a 10-h night at 20°C). Arabidopsis and *N. benthamiana* plants were watered with a complete nutrient solution as previously described [43].

The *lcnp* KO mutant line (*lcnp-1*) was available through the European Arabidopsis stock Center at Nottingham University. The *LCNP* overexpressing line was provided by Dr F Ouellet from Université du Québec (Montréal, Canada).

**Chlorophyll Fluorescence Measurements**

Chlorophyll fluorescence from leaf disks was measured at room temperature using a PAM-2000 modulated fluorometer (Walz, http://www.walz.com) as previously described [25]. The maximal quantum yield of photosystem II photochemistry was measured as

\[
F_v/F_m = (F_m - F_0) / F_m
\]

where \(F_m\) is the maximal fluorescence level (measured using a 800 msec pulse of saturating light) and \(F_0\) is the initial fluorescence level (measured after a 2 sec pulse of far-red light).

For the comparison of the different experimental groups, statistical differences of means were analyzed with the Student’s t or ANOVA tests. Letters have been used to mark statistically identical groups of means. P values are given in the legend of figure for each group of means.
Chlorophyll fluorescence imaging was done with a laboratory-built instrument as described by Johnson et al. [44].

**RT-PCR**

Total RNA extraction, cDNA preparation, and RT-PCR were performed as previously described [45]. Expression of APOD was analyzed by RT-PCR with specific primers TepGW and GWapoD (Additional file 1). As control, RT-PCR was performed using actin gene-specific primers (At2g37620, Additional file 1).

**Construction of Transgenic Plants**

DNA sequences, which encode human APOD and the putative transit peptide of LCNP from Arabidopsis (At3g47860) were amplified by polymerase chain reaction (PCR). The primers used for PCR are indicated in Fig. 1A, their sequences are listed in Additional file 1. The final PCR product displayed attB recombination sites, which allowed cloning in pDONR201 vector using a Gateway BP Clonase enzyme mix (Invitrogen). One entry clone was fully sequenced before subsequent cloning in the binary Gateway destination vectors pB2GW7 and pB7FWG2 (Plant Systems Biology, VIB-Ghent University, Belgium; [46]) by using a Gateway LR Clonase enzyme mix (Invitrogen). The pB2GW7 vector allows expression of the cloned sequence and pB7FWG2 vector allows fusion of EGFP with the APOD C-terminus. For both vectors, the cDNA is placed under the control of the 35S CaMV promoter and of the 3′ untranslated transcribed region of a nopaline synthase gene. The binary constructs were introduced into the *Agrobacterium tumefaciens* strain C58. The resulting bacterial culture containing the recombinant pB2GW7 was used to transform Arabidopsis ecotype Columbia-0 (Col0) the standard flower dip method [47]. Transgenic plants were selected in soil after Basta treatment. The recombinant Agrobacterium strain containing the pB7FWG2 construct was infiltrated in *Nicotiana benthamiana* leaves according to the protocol described by Leuzinger et al. [48].
**Chloroplast Purification and Chlorophyll Measurement**

Intact chloroplasts from Arabidopsis leaves were extracted and purified by isopycnic centrifugation according to Seigneurin-Berny et al. [49]. All operations were carried at 5°C. In brief, Arabidopsis leaves (100 g, from 5 week-old plants) were ground in 400 mL of grinding medium (20 mM, Tricine, pH 8.4 containing 0.4 M, Sorbitol; 10 mM, Na$_2$-EDTA; 10 mM, NaHCO$_3$; 0.1% [w/v] Bovine serum albumin) using a Waring blender. Chloroplasts were collected by low speed centrifugation at 2070 x g for 2 min. The chloroplasts in the pellet were re-suspended in washing buffer (20 mM, Tricine, pH 7.6; 0.4 M, Sorbitol; 2.5 mM, MgCl$_2$; 1.25 mM, Na$_2$-EDTA) and loaded on the top of a preformed continuous Percoll gradient. Intact chloroplasts, which concentrated in a green band at the lower part of the gradient were recovered and rinsed with washing buffer. The upper part of the gradient contains broken chloroplasts. Following low speed centrifugation (2070 x g for 2 min), chloroplasts were osmotically lysed in 10 mM Tris-HCl, pH 8. The thylakoid membranes were collected by centrifugation. Stromal proteins, which remained in the supernatant were acetone precipitated. Thylakoids were resuspended in hypotonic buffer containing 200 mM NaCl and finally concentrated by low speed centrifugation.

Chlorophyll was extracted with 80 % [v/v] aqueous acetone and quantified by spectrophotometry following the Arnon’s method.

**Protein Extraction, SDS-PAGE and Western Blotting**

Preparation of a total protein extract from Arabidopsis leaves was performed according to Rumeau et al. [42]. Following grinding in liquid nitrogen, the powder was resuspended in extraction buffer [10 mM Tris-HCl pH 8 containing 20 mM NaCl, 2 mM Na$_2$-EDTA, 5 mM Dithiothreitol]. The extract was filtrated trough two layers of muslin and the proteins were acetone [80 %; v/v] precipitated. Denaturing SDS-PAGE was performed as described by Laemmli [45] using 13 % acrylamide gels. Proteins were either stained with BioSafe Coomassie
(BioRad) or electro transferred onto 0.45 µm nitrocellulose membrane (Biotrace Pall) and probed with antibodies. Antibodies against APOD were purchased at Sigma-Aldrich/Merck. Immunocomplexes were visualized with 1:5000 diluted anti-rabbit IRDye800 antibody (Invitrogen). Detection was enabled by the Odyssey infrared imaging system.

**Confocal Microscopy Observation**

Pieces of *N benthamiana* leaves were sampled and mounted in water. Imaging was performed using a Zeiss LSM 780 confocal laser scanning microscope and either a 20x (Plan-Apo) or a 40x (C-Apo Corr FCS) objective. For EGFP and mCherry fluorescence analysis, the 488 nm excitation line of an argon laser was used and the fluorescence signal was detected using an emission band width of 490-530 for EGFP and of 590-620 for mCherry.

**3D Structure Prediction and Structural Alignment**

Three-dimensional structure of LCNP was predicted and modeled from Phyre² database [51]. The top model with 100 % confidence was considered. Structural alignment with the human APOD model (PDB: 2HZR; [30]) was achieved using the Matchmaker tool in the UCSF Chimera package [52].

**Abbreviations**

ABA: Abscisic acid; APOD: Apolipoprotein D; CaMV: Cauliflower Mosaic Virus; EGFP: Enhanced Green Fluorescent Protein; ER: Endoplasmic Reticulum; Fv: Variable fluorescence; Fm: Maximal Fluorescence; KO: Knock-Out; LCNP: Plastid Lipocalin; LHCII: Light Harvesting Complex II; MetSO: Methionine Sulfoxide; NPQ: non-photochemical quenching; O/E: Over Expression; PAGE: Polyacrylamide gel electrophoresis; PCR: polymerase chain reaction; PQ: Paraquat (N,N’-dimethyl-4-4’-bipyridinium dichloride); PSII: Photosystem II; ROS: Reactive Oxygen Species; RT-PCR: Reverse Transcription Polymerase Chain Reaction;
**Declarations**

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**Ethics approval and consent to participate**

Not applicable

**Consent for publication**

Not applicable

**Availability of data and material**

Not applicable

**Competing interests**

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

**Authors' contributions**

DR conceived the project and designed the experiments. DR and PH performed the experiments. DR wrote the manuscript and PH read and approved it.

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Figure Legends

**Figure 1. Molecular characterization of transgenic plants expressing a chimeric gene containing the LCNP putative transit peptide fused to APOD cDNA.** A, The gene structure. Shaded and white box represent the sequences coding for APOD and LCNP transit peptide (TP), respectively. Small arrows represent PCR primers whose sequences are listed in Additional file 1. In the lower part, amino acid sequence of the chimeric protein is also indicated. In bold, LCNP transit peptide. B, RT-PCR analysis of transgenic plants. Total RNA was extracted from leaves and PCR was performed using TPeGW and GWapoD as gene-specific primers for the chimeric gene (left panel) and actin gene as an internal control [right panel]. C, Western blot analysis. Total proteins were extracted from leaves and subjected to gel blot analysis using anti-APOD antibodies. Inset, Coomassie blue stained gel as a loading control.

**Figure 2. Subcellular localization of APOD.** Intact chloroplasts were isolated from leaves of the a661 transgenic line by centrifugation through a Percoll gradient. Chloroplasts were lysed by hypo osmotic shock and further separated into thylakoid and stroma fractions for immunoblot analysis. Thylakoids were washed with a buffer containing 200mM NaCl. Proteins collected following acetone precipitation were separated on a 13% SDS-PAGel. A, Coomassie Blue stained gel; B, immunoblot detection of APOD in the chloroplast sub fractions.

**Figure 3. Co-localization of APOD-EGFP with compartmental markers.** *Nicotiana benthamiana* leaves were agroinfiltrated for the transient expression of APOD:EGFP. Confocal scanning microscopy observations were performed at 6 days post infiltration. A, Chloroplast marker chlorophyll autofluorescence; B, Endoplasmic reticulum marker m Cherry-CN1; C,
Golgi marker GNT1-mCherry. (s, stomata). N. benthamiana infiltrations with the different constructs were reproduced several times and gave similar results.

**Figure 4. Tolerance of Arabidopsis APOD-containing transgenic lines to water deficit stress.**

Nine plants of each line were grown in a single pot containing a mix of soil and sand (2/1, w/w) under normal conditions for 3 weeks. Then watering was completely withdrawn for 10 days [A]. Plants were then re-watered for 5 days [B]. These experiments were repeated twice and gave similar results.

**Figure 5. Tolerance of Arabidopsis APOD-containing transgenic lines to oxidative treatment.** Leaf disks were floated on a solution of paraquat [PQ, 1 μM] for 8 hours and exposed to light (130 μmol m^{-2} s^{-1}). The photosystem II photosynthetic efficiency in the leaf disks was estimated by chlorophyll fluorescence determination of photochemical yield (Fv/Fm). The data represent the means ± SD (n>6). Student's t- and ANOVA-tests performed, enabled us to identify 4 groups of statistically different means (P ≤ 0.001). P values of "t" tests of groups b, c and d were 0.560, 0.977 and 0.559, respectively.

**Figure 6. Fluorescence phenotype in APOD-containing transgenic lines induced by cold and high light treatment.** Isolated leaves were exposed to cold (6°C) and high-light (1500 μmol photons m^{-2} sec^{-1}) treatment for 3 hours. Representative image of maximum chlorophyll fluorescence emission (Fm) monitored with a fluorescence imaging system.
Figure 7. Overlay of the protein structures of human ApoD (purple ribbon) and the homology model of LCNP (blue ribbon). The methionine residues in both molecules are highlighted in black. The backbone rmsd is 1,117Å between the mean structures indicating that the global folds are similar for both proteins despite a low sequence identity (31% identity, 100% confidence, data not shown).

Additional file 1. Sequences of PCR primers designed for the cloning of chimeric genes and transgenic plants characterization.

TepGW
GGGGACAAGTTTGTACAAAAAAGCAGGCTTCATGATATTATTAAGTAGTAGTATA
AGC
TepTil2 AACTAGGGAGTGCTGCTGCTACAGAGAAGAGGATAGGAA
TilPep2 TTCCATCTCTTTCTTCTGTAGCAGCAGCAGACTCCCTAGTT
GWTilHA:GGGGACCACCTTTGTACAGAAGAGCTGGGTATTACGAGAGCTTGGGGCAGTTCAC
ATCGTATGGGTATTTTGCCGAAGAGAGATTTGAACCAC
Tpep long apoD  AACTAGGGAGTGCTGCTGCTCAAGCATTTCATCTTGGGAA
apoD Tpep long  TTCCCAAGATGAAATGCTTGAGCAGCAGCAGACTCCCTAGTT
GWapoD
GGGGACCACCTTTGTACAGAAGAGCTGGGTATTACGAGAGCTTGGGGCAGTTCAC
REFERENCES


6- Bugos RC, Hieber AD, and Yamamoto HY. Xanthophyll cycle enzymes are members of the lipocalin family, the first identified from plants. J Biol Chem. 1998;273:15321-15324.


Arabidopsis lipocalins AtCHL and AtTIL have distinct but overlapping functions essential for lipid protection and seed longevity. Plant, Cell & Env., 2014;37:368-381.


31- Vogt M, Skerra A. Bacterially produced apolipoprotein D binds progesterone and arachidonic acid, but not bilirubin or E-3M2H. J Mol Recognit. 2001;14:79-86.


Figure 1

A  TP-APOD construction

MILLSSSILSRPVS5QSFSPPAAATSTRSHSVTVKCCCSSRRLLKNPEKCSLENLEIQALRKCF VSGFAAILLLSQAGGIADLSGGYQNICQLGSAAAQAFHLGKCPNPVQENFDVNKYLGRWYEI EKIPITFENRGCIQANYSLMENGKIKVNLQERADTVNGIJEATPVNLTEPAKLEVKFSWFMP SAPYWILATDYENYALVY5CTCIQLFHVDFAWILARNPNLPPETVDSKLNTSNNIDVKKMTVD QVNCPKLS

B  RT-PCR

C  Western blot: Ab ApoD
Figure 2

SDS-PAGE

Western Blot
Figure 3

A  Bright  EGFP  chlorophyll  overlay

B  EGFP  CNX1-Cherry  overlay

C  EGFP  GNT1-Cherry  overlay
Figure 4
Figure 5

Fv/FM

- Co10
- Co10 + PQ
- lcnp
- lcnp + PQ
- LCNP O/E
- LCNP O/E + PQ
- a661
- a661 + PQ
- a421
- a421 + PQ
- a212
- a212 + PQ
Figure 6

Col0      LCNP
O/E   lcnp  soq1

Fm

Col0  a661  a421  lcnp
Figure 7
### Supplementary Table I

Supplementary Table I: Sequences of PCR primers designed for the cloning of chimeric genes and transgenic plants characterization

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