Ectopic expression of human apolipoprotein D in Arabidopsis plants lacking chloroplastic lipocalin partially rescues sensitivity to drought and oxidative stress

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Ectopic expression of human apolipoprotein D in Arabidopsis plants lacking chloroplastic lipocalin partially rescues sensitivity to drought and oxidative stress.

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Highlights

- Human *APOD* was introduced into Arabidopsis plants lacking chloroplastic lipocalin.
- APOD did not substitute to plastid lipocalin in low temperature and high light-induced generation of qH, the slowly reversible component of NPQ.
- APOD partially rescued sensitivity to oxidative stress.
- APOD partially rescued sensitivity to water stress.

Abstract

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 adverse conditions by preventing accumulation of fatty acid hydroperoxides 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 thaliana*. We introduced APOD cDNA fused to a chloroplast transit peptide encoding sequence in an Arabidopsis LCNP KO 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. 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 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. This work provides a base of understanding the molecular mechanism underlying LCNP protective function.

Keywords:

Lipocalin, Chloroplast, Apolipoprotein D, Oxidative stress, Arabidopsis

Abbreviations:

ABA, Abscisic acid; APOD, Apolipoprotein D; CaMV, Cauliflower Mosaic Virus; CNX1, Calnexin 1; EGFP, Enhanced Green Fluorescent Protein; ER, Endoplasmic Reticulum; Fv, Variable fluorescence; Fm, Maximal Fluorescence; GNT1, N-acetylglucosaminyltransferase 1; KO, Knock-Out; LCNP, Plastid Lipocalin; LHCII, Light Harvesting Complex II; MetSO, Methionine Sulfoxide; NPQ, non-photochemical quenching; O/E, Overexpression; 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; SDS, Sodium dodecyl sulfate; TBARS, Thiobarbituric acid-reactive substances; TIL, Temperature-Induced Lipocalin; VDE, Violaxanthin-de-Epoxidase; ZE, Zeaxanthin Epoxidase.

1. Introduction

Lipocalins constitute an evolutionary conserved family of small proteins widely distributed in nature whose common feature is their ability to bind small hydrophobic molecules (Flower 1996). 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 (Akestrom et al. 2000). 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 (Flower 1996; Schiefner and Skerra 2015). 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 (Bratt 2000; Xu and Venge 2000), human and animal lipocalins are extensively studied.

Genomic data mining demonstrated that plants also possess lipocalins, which were classified as temperature-induced lipocalin (TIL) and chloroplastic lipocalin (LCNP[1]) (Charron et al. 2005). 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 (Chi et al. 2009), its accumulation during cold acclimation was described in Arabidopsis leaves (Kawamura et Uemura 2003) and in Siberian spruce needles (Kjellsen et al. 2010). *TIL* gene expression has been demonstrated to increase with heat and cold treatment in wheat and Arabidopsis (Charron et al. 2002) and peach fruits (Zhang et al. 2010). In *Oryza sativa*, a TIL encoding gene was reported to be highly up regulated under heat stress in panicles of tolerant rice cultivar (Prasanth et al. 2017). *TIL* expression was also significantly induced by a cold treatment in *Medicago sativa* subsp. *falcata*, a forage legume cold tolerant (He et al. 2015) and in a grapefruit following a conditioning treatment that enhances chilling tolerance (Maul et al. 2011). In grape berries of *Vitis labruscana*, salicylic acid treatment up-regulated *TIL* preventing postharvest loss during cold storage (Cai et al. 2014). In mango fruits a TIL homolog and its corresponding gene appeared to be up-regulated during a brassinolide-mediated response to cold stress (Li et al.

[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.

2012). Moreover, TIL plays a role in salt tolerance in *Populus euphratica*, a salt-tolerant poplar species (Brinker et al. 2010; Abo-Ogiala et al. 2014). In tomato two TIL encoding genes have been characterized, they display different expression profiles during development and might have a role in oxidative stress response (Wahyudi et al. 2018).

Considering LCNP, it has been demonstrated that high light, dehydration, oxidative stress and abscisic acid (ABA) induced transcript and protein accumulation in Arabidopsis (Levesque-Tremblay et al. 2009). In wheat, cold acclimation induced *LCNP* mainly in freezing-tolerant cultivar while heat exposure down-regulated gene expression (Charron et al. 2005). In *Festuca arundinacea*, salt stress induced a LCNP protein level increase, which was higher in a salt-tolerant genotype (Pawlowicz et al. 2018). Furthermore, LCNP abundance similarly decreased in response to drought treatment in two cultivars of *F arundinacea*, however, after re-watering lipocalin level returned to initial level only in the high-drought-tolerant genotype (Kosmala et al. 2012).

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 hydroperoxy fatty acids and thus stop the lipid peroxidation chain reactions initiated by reactive oxygen species (ROS) generated during stresses (Charron et al. 2008; Chi et al. 2009). In 2009, Levesque-Tremblay et al. 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. Bocca et al. (2014) 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. (2018) 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 (Malnoë et al. 2018).

Phylogenetic studies established that plant lipocalins share similarities with the bacterial lipocalin, the mammalian apoliprotein 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 (Ganformina et al. 2008). Conversely, human *APOD* overexpression reduced lipid peroxidation in mice and Drosophila (Ganfornina et al. 2008; Muffat et al. 2008). Increased APOD levels in the human brain were reported under conditions that promote lipid peroxidation such as aging or Alzheimer's disease (Terrisse et al. 1998). At the biochemical level, APOD ligands are well-known (Rassart et al. 2000; Vogt and Skerra 2001; Eichinger et al. 2007) 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 (Bhatia et al. 2012). Insofar as LCNP displays structure conservation with APOD and displays similar antioxidative function, we wanted to investigate whether the lack of the chloroplastic LCNP could be

offset by human APOD whose molecular properties are already established. 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 oxidative and water-tolerant phenotype suggesting that human APOD can replace in part, LCNP. However, when investigating APOD function in thylakoids after high light stress at low temperature, it appeared that APOD could not contribute to qH, the slowly reversible form of non-photochemical chlorophyll fluorescence quenching, as described for LCNP. Reasons for this partial complementation are discussed and possible directions for future research are also proposed.

2. Materials and Methods

2.1. Plant Materials, 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 (Rumeau et al. 2005).

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).

2.2. 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 (Malnoë et al. 2018). 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. (Johnson et al. 2009).

2.3. RT-PCR

Total RNA extraction, cDNA preparation, and RT-PCR were performed as previously described (Fabre et al., 2007). 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). APOD and Actin fragments were amplified using the following parameters: initial denaturation at 94°C for 3 minutes, 27 cycles of 94°C for 30 seconds, 60°C for 20 seconds and 72°C for 30 seconds, followed by a final extension at 72°C for 2 minutes.

2.4. 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; Karimi et al. 2002) 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 (Clough et al. 1998). 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. (2013).

2.5. Chloroplast Purification and Chlorophyll Measurement

Intact chloroplasts from Arabidopsis leaves were extracted and purified by isopycnic centrifugation according to Seigneurin-Berny et al. (Seigneurin-Berny et al. 2008). 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.

2.6. Protein Extraction, SDS-PAGE and Western Blotting

Preparation of a total protein extract from Arabidopsis leaves was performed according to Rumeau et al. (2005). 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 (1970) 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.

2.7. 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.

2.8. Lipid peroxidation assay

The level of lipid peroxidation products was estimated using the thiobarbituric acid (TBA) test, which determines thiobarbituric acid-reactive substances (TBARS) (Heath and Packer 1968). TBARS content was spectrophotometrically determined in Arabidopsis leaf disks (200 mg) from wild-type, transgenic and mutant plants as reported by Sunkar et al. (2003).

3. Results

3.1. 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. The 20-residu APOD signal peptide, which is processed in the human mature APOD form was removed in the plant construct (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 (Levesque-Tremblay et al. 2009). Primary transgenic Arabidopsis plants were identified following Basta selection 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 a421) 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 (Levesque-Tremblay et al. 2009). 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. Its concentration increased only slightly in the chloroplast fraction, probably because the enrichment of proteins from percoll-purified chloroplasts in this fraction compared with total protein was low. However, we observed a larger amount of APOD in both chloroplast compartments, thylakoids and stroma. 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 *N. benthamiana* leaves (Fig. 3). Six days later, leaf epidermal cells were observed by confocal microscopy. EGFP fluorescence coincided strongly with chlorophyll autofluorescence (Fig. 3A and 3B) 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. 3B). Furthermore, EGFP fluorescence was also detected in other subcellular structures throughout the cell (Fig. 3A). To identify EGFP labeled structures co-localization experiments were performed with different compartment markers. Constructs containing mCherry fused either to calnexin1 (CNX1) or N-acetylglucosaminyltransferase1 (GNT1) were used as markers of endoplasmic reticulum network (ER) and Golgi, respectively (Batistic, 2012, Fig. 3C and 3D). Fluorescence was evident in the ER and in Golgi. E-GFP fluorescence was also detected in nuclei (Fig 3D) but only in a few cells suggesting that it is non-specific signal. Indeed it has been reported that GFP may translocate to the nucleus due to unspecific diffusion (Seibel et al. 2007). In contrast co-localization experiments performed with a marker of plasmalemma and tonoplast excluded the presence of proteins in these membranes (data not shown).

3.2. Effect of APOD expression on sustained photoprotective energy dissipation

Recently, it has been clearly 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 (Malnoë et al. 2018). Whether APOD can replace LCNP in this function was investigated. Detached leaves of transgenic plants expressing *APOD* 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, wild-type plants (Col0) and the different

mutant and transgenic lines displayed similar maximal fluorescence parameter (Fm, data not shown). After stress (Fig. 4), as expected since already described by Malnoë et al. 2018, *soq1* displayed a lower Fm compared with Col0 while Fm in *lcnp* was higher than in the wild-type 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.

3.3. 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 (Levesque-Tremblay et al. 2009). 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 oxidative stress (Fig. 5) and drought (Fig. 6).

Since previous studies indicated that LCNP is involved in the protection of the photosynthetic apparatus against ROS, we first 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 wild type (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.

A protective effect of APOD against a drought stress was also investigated. 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 overexpressing *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.

3.4 Effect of APOD expression of lipid peroxidation in Arabidopsis plants submitted to an oxidative stress

Numerous animals and human studies have reported increase APOD expression in response to conditions that promote lipid peroxidation (Terrisse et al. 1998; Ordoñez et al. 2006; Hou et al. 2016). Moreover, it has been

demonstrated that APOD functions to protect against lipid peroxidation and oxidative stress (Ganfornina et al. 2008; Muffat et al. 2008). Its capacity to scavenge hydroxyl radicals (Zhang et al. 2018) and catalyze the reduction of hydroperoxides of arachidonic acid to the non-reactive alcohols, thereby preventing lipid peroxidation propagation by radical-driven chain reactions (Bhatia et al. 2012) has also been established.

In order to further study the beneficial role of APOD in Arabidopsis plants submitted to abiotic stresses and investigate a possible function in protecting against lipid peroxidation, Arabidopsis leaf disks were exposed to a PQ-induced oxidative stress as described in Fig. 5 [1µM PQ for 8 hours and exposed to light (130µmol m-2 s-1)]. The levels of lipid peroxides in plant tissues were determined as TBARS contents (Fig. 7). TBARS result from the reaction of peroxidized polyunsaturated fatty acids with thiobarbituric acid. Thus, they are an index of lipid peroxidation and have been used as a metabolic marker for oxidative stress (Heath and Packer, 1968). As shown in Fig.7, PQ treatment induces an increase in the TBARS content. *Lcnp* leaf disks accumulate a significantly higher amount of TBARS compared to the control (Col0) when exposed to the stress. Lower amounts of TBARS were observed in *LCNP O/E* and *APOD* (a421 transgenic line) treated disks indicating that the degree of lipid peroxidation was lower in these plants. However, APOD samples displayed higher TBARS level than LCNP O/E disks. These results suggest that in Arabidopsis, APOD could exert a reductive activity on plant lipid hydroperoxides and thus prevent lipid peroxide propagation as described in animals and humans. However, this effect is not as effective as that of LCNP. This result is consistent with the effect of PQ on Fv/Fm described in Fig. 5. As previously proposed for LCNP (Levesque-Tremblay et al. 2009), the lower PSII damage observed in APOD transgenic plants following an oxidative stress is probably due to the ability of APOD to alleviate lipid peroxidation in Arabidopsis.

Discussion

In the present work, we developed an interkingdom complementation approach to investigate Arabidopsis chloroplastic lipocalin function. The rationale behind this approach was the assumption that human APOD because it shares similar antioxidative properties and a similar three-dimensional structure with LCNP can rescue the lack of chloroplast lipocalin in Arabidopsis. Numerous reports have demonstrated that interspecies functional complementation studies facilitate structure-function analysis (Dolinski and Botstein, 2007; Schultz and Thöny-Meyer, 2000). To the best of our knowledge, only one report addressed a functional complementation of plant protein with animal protein (Brioudes et al. 2010). Here, 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 (Malnoë et al. 2018). It is intriguing to notice that in Arabidopsis, APOD can fulfill only certain functions of LCNP.

The appropriate subcellular localization and translocation of lipocalins are crucial to their functionality. LCNP located into the lumen of thylakoids (Levesque-Tremblay et al. 2009) 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 (LHCII) membrane environment (Malnoë et al. 2018). Lumen targeting is a complex process, which may involve two different pathways able to recognize a thylakoid-targeting signal peptide (Albiniak et al. 2012). 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 communication). 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 (Faye and Daniell, 2006) but import into thylakoids lumen has never been described. The lack of lipocalin into the chloroplast subcompartment, although the protein is present in the stroma, may explain why APOD did not contribute to qH as LCNP does.

Moreover, it has been demonstrated that an unpaired cys residue in APOD is responsible for covalent attachment of the protein with Apolipoprotein A-II (APOA-II) in plasma (Vogt and Skerra, 2001). Although no homolog of APOA-II has been described in plants and since the cys residue was maintained in the plant chimeric construct we cannot exclude unphysiological crosslinking of APOD to membrane proteins in plant cells. This could account for the localization of EGFP-APOD in ER and Golgi (Fig. 3).

Molecular phylogenetic analysis of lipocalins not only revealed that the chloroplastic LCNP shared significant structural homology with the mammalian APOD (Ganfornina et al. 2000; Charron et al 2005) 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 (Charron et al. 2005; Lakshmi et al. 2015). *In vitro* ligand binding assays have shown that APOD is able to bind a variety of lipids in its hydrophobic binding cavity (Rassart et al. 2000). More recently, it has been clearly demonstrated that APOD displayed the highest binding affinity for arachidonic acid (AA) and progesterone (Rassart et al. 2000; Vogt and Skerra 2001; Eichinger et al. 2007). 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 Dassati et al. 2014). In 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 published 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 (Terrisse et al. 1998; Ordoñez et al. 2006; Hou et al. 2016). Studies in mice showed that loss of APOD increased lipid peroxidation in the brain, that cerebral APOD expression is increased in response to PQ-induced oxidative stress and that human APOD expression reduced lipid peroxidation (Ganfornina et al. 2008; Muffat et al. 2008). In addition, expression of human APOD reduced the accumulation of aldehydic end-products of lipid peroxidation in an aged Drosophila model (Walker et al. 2006; Muffat et al. 2008). 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 (Levesque-Tremblay et al. 2009). Thus, a LCNP KO Arabidopsis line, which exhibited serious damage upon photo-oxidative stress induced by drought, high light or PQ displayed a rapid accumulation of hydroperoxy fatty acids. In contrast, in LCNP overexpressing lines, which coped better with these conditions, the lipid peroxidation level remains very low (Levesque-Tremblay et al. 2009). Related to APOD antioxidative function, it has been demonstrated that APOD had the capacities to scavenge hydroxyl radicals (Zhang et al. 2018) and catalyze the reduction of hydroperoxides of AA to the non-reactive alcohols, thereby preventing lipid peroxidation propagation by radical-driven chain reactions (Bhatia et al. 2012). This reaction involves a highly conserved methionine residue (Met93 in human APOD) located at the hydrophobic surface of the protein and results in oxidation of the methionine thioether group to form methionine sulfoxide (MetSO, Bhatia et al. 2012). Interestingly, APOD expression in Arabidopsis decreases PQ-induced lipid peroxidation (Fig. 7) suggesting that in plants APOD could scavenge hydroxyl radicals and limit lipid peroxidation. Thus, 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. 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 conclusion, our data indicate that despite structural homology and similar contribution to lipid oxidation status in cells, APOD when introduced in Arabidopsis cannot fully substitute for LCNP. We cannot exclude that APOD and LCNP share similar oxidative protecting mechanisms, however, APOD displays molecular features which hamper its protective function in plant. Thus, we propose that APOD may be glycosylated and able to bind other proteins in plant cell and thus unable to reach thylakoid lumen where LCNP plays its photoprotective role. APOD that remains in the cytosolic compartment is able to partially protect cells from reactive oxygen species produced during environmental stress. We suggest that this functionality may reside in a particular methionine residue, which has been demonstrated to play a crucial role in animal APOD protective activity. 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. Finally, we also intent to change the unpaired APOD Cys to investigate whether APOD inability to protect photosystem II is due to its failure to reach the thylakoid lumen.

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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Declarations

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Not applicable

Consent for publication

Not applicable

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Conflicts of interest

The authors declare that they have no conflicts of interest.

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Color should be used for Figures 3, 4 and 6

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 Supplemental Table I. 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 TPepGW 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**, Cell expressing EGFP in various subcellular structures; **B**, Chloroplast marker chlorophyll autofluorescence; **C**, Endoplasmic reticulum marker m Cherry-CNX1; **D**, Golgi marker GNT1-mCherry; (s, stomata). *N benthamiana* infiltrations with the different constructs were reproduced several times and gave similar results.

Figure 4. ***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 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 ($P \leq 0.001$). P values of "t" tests of groups b, c and d were 0.560, 0.977 and 0.559, respectively.

Figure 6. ***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 (**A**). Then watering was completely withdrawn for 10 days (**B**). Plants were then re-watered for 5 days (**C**). These experiments were repeated twice and gave similar results.

Figure 7. ***APOD expression reduces paraquat-induced lipid peroxidation in transgenic plants.*** 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). Lipid peroxidation is expressed as TBARS contents. Values are given as means ± SD from three replicates and each letter represents a statistical different group ($P<0.001$).

Supplementary Table I. Sequences of PCR Primers designed for the cloning of chimeric genes and transgenic plant characterization.

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TepGW
ggggacaagtttgtacaaaaaagcaggcttcatgatattattaagtagtagtataagc
TpepTil2
aactagggagtgctgctgctacagagaagaaagagatggaa
TilPep2
ttccatctctttcttctctgtagcagcagcactccctagtt
GWTilHA
ggggaccactttgtacaagaaagctgggtattaagcgtaatctggaacatcgtatgggtatttgccgaagagagatttgaaccacc
Tpep long apoD
aactagggagtgctgctgctcaagcatttcatcttgggaa
apoD Tpep long
ttcccaagatgaaatgcttgagcagcagcactccctagtt
GWapoD
ggggaccactttgtacaagaaagctgggtattacgagagcttggggcagttcac
Actin Dir
aaaatggctgatggtgaagaca
Actin Rev
gatggttatgacttgtccat