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The role of plastoglobules in thylakoid lipid remodeling during plant development☆

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A B S T R A C T

Photosynthesis is the key bioenergetic process taking place in the chloroplast. The components of the photosynthetic machinery are embedded in a highly dynamic matrix, the thylakoid membrane. This membrane has the capacity to adapt during developmental transitions and under stress conditions. The galactolipids are the major polar lipid components of the thylakoid membrane conferring bilayer properties, while neutral thylakoid lipids such as the prenyllipids and carotenoids contribute to essential functions such as electron transport and photoprotection. Despite a large number of studies, the intriguing processes of thylakoid membrane biogenesis and dynamics remain unsolved. Plastoglobules, thylakoid-associated lipid droplets, appear to actively participate in thylakoid function from biogenesis to senescence. Recruitment of specific proteins enables the plastoglobules to act in metabolite synthesis, repair and disposal under changing environmental conditions and developmental stages. In this review, we describe plastoglobules as thylakoid membrane microdomains and discuss their involvement in lipid remodeling during stress and in the conversion from one plastid type to another. This article is part of a Special Issue entitled: Chloroplast Biogenesis.

Keywords:
Plastoglobule
Lipid droplet
Thylakoid membrane
Lipid remodeling
Microdomain
Plastid differentiation

1. Introduction

During the last 1.5 billion years, plastids evolved from cyanobacteria to become the most versatile organelle in plant cells [1,2]. In angiosperms, totipotent proplastids are maternally inherited (but exceptions exist) [3–5]. They are able to differentiate into a large diversity of functional entities, from light harvesting photosynthetic factories to starch storage sites [6]. Depending on tissue, developmental stage and environmental conditions, proplastids morph into etioplasts, chloroplasts or leucoplasts, while chromoplasts and gerontoplasts are normally derived from chloroplasts [7]. Even conversions of differentiated plastids into another plastid type are possible and require extensive ultrastructural reorganization [8]. In this review, we will focus on chloroplasts and three developmentally related forms, namely etioplasts, chromoplasts and gerontoplasts.

Etioplasts occur in photosynthetic tissues in the absence of light and represent the chlorophyll-less chloroplast precursor. Chromoplasts are found in reproductive tissues and lend attractiveness to pollinators and disseminators by storing colored carotenoids. Gerontoplasts represent senescent chloroplasts and appear during leaf senescence. Highly regulated catabolic activities take place in gerontoplasts, while resources are relocalized to seeds or perennial tissues.

Chloroplasts emerge upon illumination (photomorphogenesis) [9]. Characterized by the presence of chlorophyll and a complex network of internal membranes, the thylakoids, chloroplasts allow plants to convert sunlight into chemical energy by photosynthesis. The light reactions of photosynthesis occur at the thylakoid membrane, a highly dynamic lipid matrix that must continuously adapt to changing environmental conditions and developmental cues.

The thylakoid bilayer is composed of four glycerolipids [10]. These include two galactolipids, monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG); one sulfolipid, sulfoquinovosyldiacyl glycerol and one phospholipid, phosphatidylglycerol. Beside these polar lipids, the thylakoid membrane also contains neutral lipids. These play essential roles in electron transport between photosystem II (PSII) and cytochrome b_6f (plastoquinone, [11]) and within PSI (phylloquinone), others act as photoprotective agents at the membrane level (tocopherol) or protect the photosynthetic complex by non-photochemical quenching (NPQ) (xanthophylls).

The thylakoids consist of a lipid bilayer enclosing a lumen, resulting in disc-shaped "sacs". The majority of thylakoids are stacked (granal thylakoids) and interconnected by non-stacked lamellae (stromal thylakoids) [12]. At the curved regions of the thylakoid membrane, globular lipid droplets, named plastoglobules (PG), are present [13]. PG were discovered by electron microscopy owing to their highly osmiophilic properties that are due to the presence of unsaturated lipids [14]. They consist of a membrane lipid monolayer surface surrounding a core of neutral lipids. This membrane lipid surface is studded with proteins [15], many of which are implicated in lipid metabolism [16,17] (Fig. 1).

Since the characterization of PG proteomes from *Arabidopsis* chloroplasts and red pepper chromoplasts, the concept has emerged that PG

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are not only implicated in the storage of neutral lipid molecules but actively contribute to their synthesis [18–20]. Around 30 proteins are associated with the PG polar monolayer (Table 1, see [20] for a complete list).

Their expression may be regulated in response to stress or developmental conditions [34]. For example, an effect of abiotic conditions, such as prolonged darkness or high light, was observed on the level of PG protein accumulation [18,25]. However, the mechanisms of their regulation at the protein level and their targeting to PG remain largely unsolved. Although none of the known PG proteins have predicted transmembrane domains, Austin et al. (2006) showed that part of the PG-localized tocopherol cyclase VTE1 (vitamin E-deficient 1) is exposed at the PG surface while another extends across the polar lipid monolayer, allowing direct contact with the neutral lipid content of the PG [13, 35,36]. PG proteins belong to three different functional groups: (1) presumed structural proteins called fibrillins (FBN) or plastoglobulins; (2) enzymes implicated in lipid metabolism and (3) uncharacterized proteins. Proteins are recruited to the PG in response to stress or developmental stage. Changes of the PG protein composition may directly and indirectly affect PG lipid composition, because several prenyllipid biosynthetic enzymes and regulatory kinases are located in PG.

It has been suggested that the membrane curvature facilitates PG formation whereby the PG stay physically connected to the thylakoid membrane through the outer membrane lipid leaflet of the thylakoid membrane [13]. Moreover, a specific bilayer composition might influence the formation of PG as it has been proposed for lipid droplets (LD). LD are structurally related to the PG and arise at the endoplasmic reticulum membrane. LD serve as lipid reservoir and site of lipid metabolism and are essential in membrane formation and maintenance [37]. The molecular mechanisms of LD formation are still under debate [38]. One of the models proposes that the products of specifically recruited neutral lipid biosynthetic enzymes accumulate between the lipid leaflets of the membrane bilayer leading to LD formation. This model is equally applicable to PG has they also host enzymes that contribute to neutral lipid biosynthesis and accumulation.

Thin layer chromatography, and recently mass spectrometry have allowed the identification of lipids in PG, but the complete lipidomic of PG is not available [22–24,26,27,33,39–43]. The neutral lipid core contains prenylquinones, carotenoids, triacylglycerol (TAG) and phytyl esters. The fact that PG and thylakoid membrane share the same kinds of neutral lipids must be emphasized even though the proportions may vary between the compartments. Concerning the polar lipid monolayer, the situation is ambiguous. In the past, galactolipids were either considered components of PG [14,44,45] or thylakoid contaminants because they were undetected in some studies but not in others [46,47]. Topologically, PG are contiguous with the outer leaflet of the thylakoid membrane bilayer and therefore logically should share the same polar lipids.

It has been proposed that the thylakoid membrane adapts to changing environmental conditions and developmental cues by lipid remodeling. Lipid turnover is essential to ensure longevity and stress resistance of membranes. Such dynamics are clearly evident in extraplastidic membranes under phosphate deficiency where a large proportion of phospholipids is replaced by DGDG [48–51].

Interestingly, Lichtenthaler in 1968 already suggested that PG have a dynamic composition according to the stage of development [44]. This hypothesis has been expanded during the last few years as it became clear that PG are sites of lipid metabolism. It can now be proposed that the PG serve to supply and maintain (neutral) lipids required for remodeling in response to environmental or developmental changes (Fig. 2).

It is therefore of high interest to elucidate the functions of PG. To achieve this goal, a wide range of methods is being used. Application of stresses and description of the resulting macro- and micro-phenotypes of plants were among the early approaches, followed by reverse genetics in conjunction with metabolomics analyses. In the following, the implication of PG in thylakoid lipid remodeling under various developmental and stress conditions will be reviewed.

2. Role of PG in plastid differentiation

2.1. Photomorphogenesis: From the etioplast to the chloroplast

In the dark or under low light intensity, proplastids develop into etioplasts, the chloroplast precursor organelles [52]. Etioplasts are characterized by the presence of the paracrystalline prolamellar body (PLB) that contains molecular building blocks for the chloroplast thylakoid membrane. Light triggers the conversion of the etioplast to the chloroplast. During this conversion, the PLB disappears while the thylakoid membrane emerges accompanied by the production of large quantities

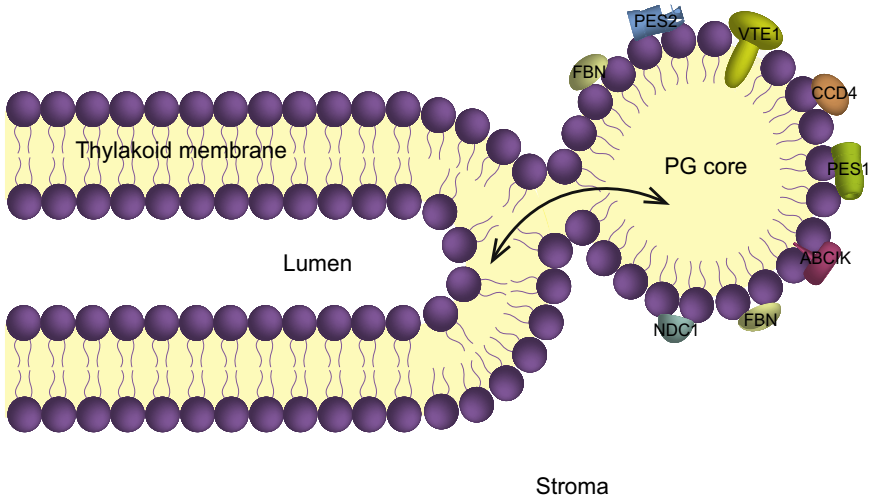

Fig. 1. Representation of the physical connection between the thylakoid membrane and the plastoglobule (PG). Within the chloroplast, the thylakoids are immersed in the stroma. The thylakoid membrane, composed of a polar lipid bilayer (mainly MGDG and DGDG), surrounds the lumen. At the curved regions of the thylakoid membrane, a PG buds from the outer thylakoid leaflet. Surrounded by a polar lipid monolayer, the PG core contains neutral lipids such as prenylquinones, carotenoids, fatty acid phytyl ester and triacylglycerol. The monolayer surface of PG is studded with specific proteins many of which are involved in prenyllipid metabolism. The contact site between PG and thylakoid membrane may provide a conduit for lipid trafficking (arrow). ABC1K, activity of bc1 complex-like kinases; CCD4, carotenoid cleavage dioxygenase 4; FBN, fibrillins; NDC1, NAD(P)H dehydrogenase C1; PES1, phytyl ester synthase 1; PES2, phytyl ester synthase 2; VTE1, tocopherol cyclase.

Table 1
Characterized plastoglobule (PG) proteins. This table provides a link between PG proteins and their roles in lipid remodeling. Despite quite numerous studies, the functions of many PG proteins remain unknown (c.f. [20] for a complete list). VTE, vitamin E-deficient; VitK, phylloquinone; PQ, plastoquinone; DGAT, diacylglycerol acyltransferase; TAG, triacylglycerol; FAPE, fatty acid phytyl ester; NCED, 9-cis-epoxycarotenoid dioxygenase; PGL, plastoglobulin; JA, jasmonate.

ID	Common name	Symbol	Function(s) in lipid metabolism	Organism	References
At4g32770	Tocopherol cyclase	VTE1	Tocopherol biosynthesis and recycling	Arabidopsis	[19,21]
At5g08740	NAD(P)H dehydrogenase C1	NDC1	VitK biosynthesis, PQ and tocopherol turnover	Arabidopsis	[16,22,23]
At1g54570	Phytyl ester synthase 1	PES1, DGAT3	TAG and FAPE biosynthesis	Arabidopsis	[24]
At3g26840	Phytyl ester synthase 2	PES2, DGAT4	TAG and FAPE biosynthesis	Arabidopsis	[24]
At4g31390	Activity of bc1-like kinase 1	ABC1K1	Regulation of prenylquinone biosynthesis	Arabidopsis	[25,26]
At1g79600	Activity of bc1-like kinase 3	ABC1K3	Regulation of prenylquinone biosynthesis	Arabidopsis	[25,27]
At4g19170	Carotenoid cleavage dioxygenase 4	CCD4, NCED4	Carotenoid oxidative cleavage	Arabidopsis	[28,29]
At4g04020	Fibrillin 1a	FBN1a, PGL35	PG structural maintenance, JA biosynthesis	Arabidopsis,	[30,31]
At4g22240	Fibrillin 1b	FBN1b, PGL33	PG structural maintenance, JA biosynthesis	Arabidopsis	[30,31]
At2g35490	Fibrillin 2	FBN2, PGL40	PG structural maintenance, JA biosynthesis	Arabidopsis	[30,31]
At3g23400	Fibrillin 4	FBN4, PGL30.4	PG structural maintenance, Stress resistance	Arabidopsis, apple	[31–33]
gi|1583601	ζ-carotene desaturase	ZDS	Carotenoid biosynthesis	Red pepper	[18]
gi|12643508	Lycopene β-cyclase	LYC-β	Carotenoid biosynthesis	Red pepper	[18]
gi|2956671	β-carotene β-hydroxylase	CrtR-β	Carotenoid biosynthesis	Red pepper	[18]

of chlorophyll. Dark-grown etiolated tissues have a pale yellow color due to the presence of carotenoids. In addition, etioplasts accumulate the chlorophyll precursor protochlorophyllide, the conversion of which to chlorophyllide and then chlorophyll occurs almost instantly in response to light [53].

Thylakoid membrane formation requires the coordinated assembly of the lipid bilayer with membrane proteins. These are mostly nuclear-encoded, imported proteins, and many are assembled to photosynthetic complexes together with cofactors such as pigments or metal ions. Galactolipids constitute 80% of the thylakoid membrane lipids. Synthesized at the inner envelope membrane, they contain fatty acids (FA) originating from the endoplasmic reticulum. FA are then incorporated into phosphatidic acid, imported into the chloroplast by the trigalactosyldiacylglycerol complex at the inner envelope and converted into diacylglycerol (DAG) [54–57]. Monogalactosyldiacylglycerol synthase 1 and digalactosyldiacylglycerol synthase 1 are required for galactolipid synthesis. Both enzymes are embedded in the inner envelope of the chloroplast and catalyze the conversion of DAG into MGDG and DGDG respectively, the two main galactolipids present in thylakoid membranes [58].

Several mechanisms of lipid transfer from the site of synthesis at envelope membrane to the nascent thylakoid membrane have been proposed. These include transport vesicles as well as membrane tubes emerging from the inner envelope or the PLB [59–64].

During chloroplast biogenesis, lamellar prothylakoids have been observed to emerge from the PLB [65,66]. The PLB itself consists of tubular structures interspersed with numerous PG [44]. Both PLB tubules and prothylakoids are composed mainly of MGDG and DGDG, the PLB being enriched in MGDG [58]. Apart from membrane lipids, the PLB also contains protochlorophyllide and proteins that are required for rapid chloroplast biogenesis. Proteomics analysis of purified PLB revealed two major functional groups of proteins: (1) Proteins implicated in pigment synthesis, such as the abundant NADPH protochlorophyllide oxidoreductases A/B and chlorophyll synthase. (2) Known thylakoid proteins implicated in photosynthetic reactions (*e.g.* ATP synthases, cytochrome b_6f, ferredoxin-NADPH oxidoreductases, PSII subunits). The protein and metabolite compositions provide the evidence that the PLB plays a role in chloroplast biogenesis and functions as a precursor of the chloroplast thylakoid membranes [52,67].

In etioplasts, the PLB cages a high number of PG. After exposure to light, PG number decreases in de-etiolated tissue simultaneously with the rise of thylakoid membranes from the PLB [44,68–70]. PG as a lipid reservoir may assist in the rapid formation of thylakoid membranes in greening tissues by releasing structural thylakoid lipids (MGDG) and their precursors (TAG, DAG). Supplying lipid building blocks for membrane expansion may explain why PG become small and rare during thylakoid formation and larger and more abundant in the mature chloroplast [44]. PG also contain tocopherol and other prenylquinones, therefore PG have also been proposed to protect the nascent membrane by supplying lipid antioxidants during chloroplast biogenesis [22].

However, two questions remain unsolved: how small hydrophobic molecules can be transferred from PG to thylakoid membrane and the extent to which PG and associated proteins are involved in thylakoid membrane formation.

While most likely the contact sites between PG and thylakoids provide the conduit for small molecules, FBN may also be involved in lipid transfer. Indeed, FBN possess a predicted lipocalin motif that has the ability to bind and transport small hydrophobic molecules [71–75]. The members of the FBN family constitute the most abundant proteins in PG. Together seven FBN (out of 13 identified in *Arabidopsis*) represent 53% of the PG proteome mass (FBN1a-At4g04020, FBN1b-At4g22240, FBN2-At2g35490, FBN4-At3g23400, FBN7a-At3g58010, FBN7b-At2g42130, FBN8-At2g46910) [20]. Initially considered structural proteins, recent studies indicate that FBN function may extend to chromoplast pigment accumulation, resistance to biotic and abiotic stresses as well as protection of the photosystem from photodamage [30,32,76,77]. These roles are in agreement with FBN expression patterns, which vary with plant developmental stage, hormonal treatment and various stresses [25,71,78–80]. Unfortunately, a PG proteome of etioplasts is still missing. Ytterberg et al. (2006) attempted to establish the PG proteome of rice etioplasts [18]. However, the isolated low-density particles are dominated by protochlorophyllide reductase and showed no overlap with other PG proteomes. For this reason, it was concluded that these low-density particles most likely represented PLB. Interestingly, a member of the FBN family was identified in these low-density particles of rice etioplasts. In *Arabidopsis*, the probable ortholog is FBN10 (At1g51110). FBN10 is mainly present in thylakoids but not particularly enriched in PG. Nevertheless, it is tempting to speculate on the implication of FBN10 in lipid trafficking during thylakoid formation.

In conclusion, the main ultrastructural modification induced by light during the transition from etioplast to chloroplast is the formation of thylakoid membrane that emerges from the PLB and is presumably assisted by the PG lipid reservoir.

2.2. Reproduction: From the chloroplast to the chromoplast

Higher plants have evolved flowers and fruits, which are specialized tissues for reproduction. Often the color of fruits and flowers are conferred by chromoplasts. Chromoplasts differentiate from chloroplasts during floral development and fruit maturation. During the process the well-developed thylakoid membrane network that characterizes chloroplasts is dismantled and replaced by carotenoid-containing structures and enlarged PG in chromoplasts [81]. During this transition, the

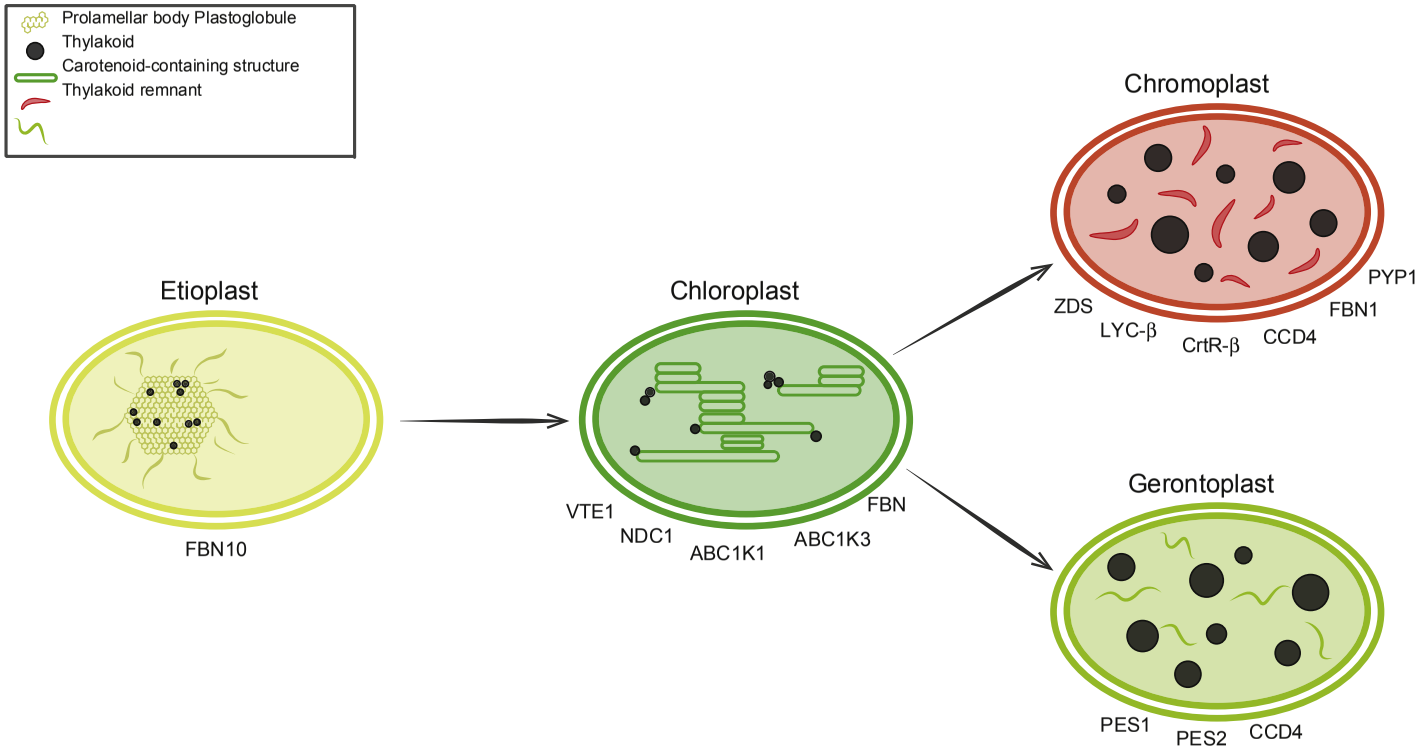

Fig. 2. Roles of plastoglobules (PG) in plastid differentiation. Etioplasts, chloroplast precursors, are present in the dark. They are characterized by the paracrystalline prolamellar body (PLB) that is interspersed with numerous small PG. As a known lipid reservoir, PG may assist in the rapid formation of thylakoid membranes upon illumination. Ytterberg et al. (2006) identified the homolog of the thylakoid-associated FBN10 in low-density lipid particles that correspond to the PLB of rice etioplasts [18]. FBN10 may serve to channel lipids to the nascent thylakoids. Chloroplasts contain the internal thylakoid membrane network. PG are coupled to the thylakoid membrane and involved in lipid remodeling. Tocopherol metabolism is upregulated under high light, involving the PG-located proteins tocopherol cyclase (VTE1), NAD(P)H dehydrogenase C1 (NDC1), activity of bc1 complex-like kinases (ABC1K) and fibrillins (FBN). Chromoplasts in reproductive tissues (fruits and flowers) differentiate from chloroplasts to attract pollinators and seed disseminators. These plastids are characterized by thylakoid disassembly and an appearance of carotenoids stored and produced in PG or carotenoid fibrils with the help of ζ-carotene desaturase (ZDS), lycopene β-cyclase (LYC-β) and two β-carotene β-hydroxylases (CrtR-β). Carotenoid cleavage dioxygenase 4 (CCD4) cleaves carotenoids to release apocarotenoids that contribute to scent and flavor. The tomato pale yellow petal 1 (PYP1) is required for xanthophyll esterification and hence normal yellow petal pigmentation. Gerontoplasts differentiate from chloroplasts during senescence. Chlorophyll and thylakoid membranes are dismantled, releasing large amounts of fatty acids and phytol. These catabolic products are sequestered in triacylglycerol (TAG) and fatty acid phytyl ester (FAPE) by phytyl ester synthase 1 (PES1) and 2 (PES2), two PG-located enzymes. TAG and FAPE contribute to the formation of giant PG. Carotenoids remaining in gerontoplasts are degraded by CCD4.

degradation of chlorophyll is concomitant with carotenoid biosynthesis and sequestration [8,82].

Carotenoids are widespread pigments synthesized and localized in plastids. They are subdivided in two classes, carotene hydrocarbons that may be cyclized at one or both ends and xanthophylls that contain extra oxygen atoms in the form of hydroxyl or epoxide groups. Carotenoids are highly esterified in fruits and flowers [83,84]. The esterification seems to enhance carotenoid stability [85] and to increase their lipophilic properties, which may facilitate their sequestration into carotenoid-containing structures [31].

During chloroplast to chromoplast conversion in red pepper, thylakoids and chlorophyll disappear whereas PG strongly enlarge. In chromoplasts, PG may transform into rod-like structures (fibrils) filled with carotenoids. The major protein associated with the fibrils was termed FBN and turned out to be the first identified member of the FBN family [31,86]. Interestingly, chromoplast fibril self-assembly occurred when FBN was added to a mixture of carotenoids and polar lipids (MGDG, DGDG and phospholipids). Fibril reconstitution only occurred when the lipid-protein ratio was close to the *in vivo* ratio. Furthermore, linear carotenoids such as lycopene inhibited fibril assembly. Cyclic carotenoids (β-carotene, zeaxanthin and capsanthin) gave intermediate results, while esterified carotenoids (zeaxanthin diester and capsanthin diester) worked best [31]. This important experiment suggested that FBN play a role in the formation and stabilization of fibrils and PG.

High expression of pepper FBN1 in tomato fruits leads to enlarged PG and protects the thylakoids from degradation during chloroplast-to-chromoplast transition [87]. Indeed, an increase in carotenoid content and a delay in thylakoid disintegration were observed under FBN overexpression. These data were interpreted in two different ways. FBN may act directly on the thylakoid membrane. Alternatively, FBN may act indirectly by enhancing PG formation and/or stabilizing their structure, including their thylakoid connections. FBN are often upregulated under abiotic and biotic stresses (for review see [71]), supporting the idea of their protective role for thylakoid membranes, be it direct or indirect.

In chloroplasts as well as chromoplasts, PG participate in carotenoid metabolism. Ytterberg et al. (2006) identified four enzymes in the PG proteome of red pepper chromoplasts that are implicated in carotenoid biosynthesis, including ζ-carotene desaturase (ZDS), lycopene β-cyclase (LYC-β) and two β-carotene β-hydroxylases [18]. ZDS introduces conjugated double bonds which are required for the biosynthesis of lycopene, the red pigment of tomato chromoplasts [88], from phytoene. LYC-β catalyzes the cyclization of one or both ends of lycopene to yield α- or β-carotene. The next step in carotenoid biosynthesis is to generate xanthophyll products by the incorporation of oxygen molecules into carotene cycles. By the action of carotenoid hydroxylases, α- and β-carotene are converted into lutein and zeaxanthin, respectively [89].

Carotenoids can also be metabolized further by oxidative cleavage to produce apocarotenoids [90]. Apocarotenoids have important metabolic functions since they act as phytohormones (abscisic acid, strigolactone), pigments (β-citraurin), aromatic volatiles (β-ionone, β-cyclocitral, geranial) or signaling molecules (β-cyclocitral). Phytohormones are essential for growth regulation, stress responses and much more [91–93]. As an aromatic volatile, β-ionone is a pollinator attractant in flowers but also contributes to fruit flavor [94]. β-Cyclocitral functions as a stress signal produced under high light. It increases the tolerance to photooxidative stress by modulating the expression of genes involved in the singlet oxygen response [95]. The oxidative cleavage of carotenoids may occur spontaneously under oxidative stress or require enzymatic activity [96]. The enzymes involved in carotenoid cleavage are grouped into the family of carotenoid cleavage dioxygenases (CCD). Members of this enzyme family have divergent roles in different tissues and species [97,98]. A subgroup termed 9-cis-epoxycarotenoid dioxygenases is essential for abscisic acid biosynthesis [99,100]. Notably, PG proteomes contain CCD4 which cleaved 8′-apo-β-caroten-8′-al to release β-ionone in *Arabidopsis thaliana* [29]. Interestingly, simultaneous absence of PG-localized ABC1K1 and ABC1K3 (activity of bc1 complex-like kinase) resulted in a threefold downregulation of CCD4, whereas protein abundance levels of several other PG-localized metabolic enzymes were not affected [25]. Thus, CCD4 stability may depend upon phosphorylation by these two PG kinases. CCD4 is a good example for the divergent roles of the CCD family even though the substrates are not fully known. The following examples strongly suggest a range of CCD4 substrates in different species.

In chrysanthemum (*Chrysanthemum morifolium* Ramat.), CmCCD4a is responsible for white color formation in petals. The expression level of CmCCD4a in yellow-flowered cultivars is extremely low, while it occurs at high levels in petals of white-flowered cultivars. Since carotenoid biosynthesis is unaffected in both cultivars, and lutein is the predominant carotenoid in yellow petals [101], it is likely that CmCCD4a cleaves lutein into colorless apocarotenoids thus disrupting the accumulation of carotenoids. Despite the fact that CmCCD4a shares 61% of homology with AtCCD4, its association with PG remains experimentally unexplored [102,103].

During Satsuma mandarin (*Citrus unshiu*) fruit ripening, the upregulation of CitCCD4 correlates with the accumulation of β-citraurin, a red pigment typical for the Yamashitabeni-wase variety. Actually, CitCCD4 was shown to cleave β-cryptoxanthin and zeaxanthin *in vitro* at the 7,8 and 7′,8′ position to yield β-citraurin. It was concluded that CitCCD4 is substrate specific since no activity was detected with lycopene, α-carotene, β-carotene and violaxanthin [104].

Heterologous expression of the saffron (*Crocus sativus*) protein shows that CsCCD4 preferentially cleaves β-carotene (but also lutein, neoxanthin and violaxanthin) into β-ionone and β-cyclocitral during stigma development. Furthermore, the expression of CsCCD4-GFP resulted in a spot-like pattern inside chloroplasts reminiscent of PG localization [105,106].

Recent insight into carotenoid sequestration stems from a study on tomato flowers [107]. The *pyp1* (pale yellow petal 1) mutant shows reduced yellow pigmentation in flowers and altered chromoplast development due to a lack of xanthophyll esters (mainly neoxanthin and violaxanthin esterified with 14:0 and 16:0 FA). Esterification enhances carotenoid stability [85] and may therefore be favored in the chloroplast-to-chromoplast transition. Interestingly, tomato PYP1 shared 58% identity (73% similarity) with phytyl ester synthase 1 (PES1) [107] which is localized in PG of *A. thaliana*. Previously named diacylglycerol acyltransferase 3 (DGAT3), PES1 contributes to TAG and fatty acid phytyl ester (FAPE) synthesis in chloroplasts [24]. Besides, its implication in carotenoid esterification is probable as PES1 was found in PG of chromoplasts of red pepper [18].

In conclusion, the chromoplast PG proteome differs strongly from that of the chloroplast [18]. It is customized to assist the chloroplast-to-chromoplast transition. This is particularly apparent by the presence of the carotenoid metabolic enzymes. These are specifically recruited for the purpose of depositing carotenoids in the emerging colored PG and fibrils.

2.3. Senescence: From the chloroplast to the gerontoplast

During senescence, nutrients are relocated from senescing tissue to the developing seeds and perennial tissues which must survive the challenges of winter [108,109]. Leaf senescence has aspects of a recycling process and is important for reproduction and plant fitness. Thus, plant senescence is a highly regulated process composed of a cascade of events, ultimately leading to cell death [110]. It is induced by external factors (*e.g.* low/high temperature, shading or lack of nutrient) or internal factors such as plant age and phytohormones. Developmental factors and stresses lead to the synthesis of jasmonate (JA), abscisic acid, salicylic acid or auxin, which initiate the transcription of genes implicated in senescence. Highly expressed during senescence, the large set of senescence associated genes plays a central role throughout senescence. A number of senescence associated genes function to catalyze catabolic

events leading to the transition from chloroplast to gerontoplast. This transition is accompanied by dramatic ultrastructural reorganization that is characterized by the dismantling of the thylakoids and the appearance of giant PG [111].

Chlorophyll breakdown is the most striking activity taking place in chloroplast to gerontoplast transition [112]. However, mutants have been identified in which chlorophyll degradation is perturbed. In the most dramatic cases this results in a stay green phenotype that may be either functional (photosynthetically active) or just cosmetic (green but photosynthetically inactive) [113].

Free phytol is a catabolic product released from chlorophyll by pheophytin pheophorbide hydrolase (PPH) [114,115]. This hydrolytic product has to be metabolized to minimize its toxicity [116]. A first pathway implicates the conversion of free phytol to phytol monophosphate by phytol kinase VTE5 followed by a second phosphorylation step and its incorporation into tocopherol [116,117]. In a second pathway, phytol is incorporated into FAPE by the esterification with free FA. These FA may either be synthesized *de novo* or result from hydrolysis of galactolipids during thylakoid dismantling. The hydrolysis of galactolipids by lipases releases a pool composed mainly of C18:3 and C16:3 free FA. FA may also be deposited in TAG.

It appears likely that the huge size of PG in gerontoplasts is at least partly due to the accumulation of these senescence-associated catabolic products. Moreover, TAG and FAPE are excellent examples to illustrate the participation of PG in chloroplast lipid remodeling during senescence both at the biochemical and ultrastructural levels. Biochemically, remodeling here refers to the synthesis of FAPE and TAG while galactolipids as well as chlorophyll are broken down. Ultrastructurally, remodeling is observed by electron microscopy: thylakoids disappear and are replaced by massively enlarged PG. Moreover, PG do not only serve to deposit FAPE and TAG but actively participate in their synthesis by the presence of three PG enzymes.

PPH (At5g13800), which dephytylates pheophytin and thereby releases free phytol, was recently identified in the PG of the *abc1k1abc1k3* double mutant lacking two PG-associated kinases [25]. The transient association of PPH with PG may be viewed as a response to the increased chlorophyll turnover observed in *abc1k1abc1k3* light-stressed leaves (see chapter 3.1).

A reverse genetic study revealed that PES1 and PES2, previously named DGAT3 and DGAT4, are overexpressed during senescence and required for FAPE and TAG production in gerontoplasts [24]. Indeed, heterologous expression in yeast and *in vitro* assays show that both proteins, members of the esterase/lipase/thioesterase family, have diacylglycerol acyltransferase activity allowing the production of TAG and FAPE from FA esterified respectively to DAG or phytol [24,110]. In addition, *pes1pes2* mutant plants under nitrogen starvation accumulate far less TAG and FAPE.

PES1 and PES2, however, are not predicted to function exclusively as acyltransferases. It is tempting to speculate that they function as galactolipid lipases at the thylakoid membrane. This idea is supported by the presence of a predicted lipase domain in both proteins, their ability to release FA from MGDG *in vitro* [24] and because the *pes1pes2* double mutant exhibits a delay in senescence characterized by a prolonged persistence of thylakoid membranes and of pale green color whereas wild type (WT) plants are yellow.

In autumn, the progressive disappearance of chlorophyll in tree leaves reveals the carotenoids that are degraded more slowly [118]. CCD4 identified in the PG proteome has recently been shown to cleave β-carotene during seed maturation and senescence [28]. Moreover, Tevini and Steinmüller (1985) observed in beech leaves (*Fagus sylvatica*) that carotenoids liberated from the thylakoid membrane were esterified and deposited in PG during senescence [46]. This suggested an implication of carotenoids in FA sequestration as carotenoid esters. This is reminiscent of the PES1 homolog PYP1 in tomato flowers that is required for normal carotenoid ester production in petal chromoplasts [107]. It therefore appears likely that PES1 and PES2 may catalyze the equivalent reaction in senescent chloroplasts.

In conclusion, the transition from chloroplast to gerontoplast occurs in close relationship with PG which serve not only to store catabolic products but also participate in their release and transformation into storage compounds.

3. Role of PG in chloroplast during stress conditions

Plants are confronted with various stress conditions, including nitrogen starvation, drought, heavy metal toxicity, temperature variations, pathogen infection and high light. In many cases, these conditions induce oxidative stress within the chloroplast.

3.1. Implication of PG in thylakoid maintenance during high light stress

Noxious reactive oxygen species (ROS) are formed during high light stress, especially singlet oxygen that is responsible for most chloroplast photooxidative damage [119]. Under high light, the major photoprotective mechanism at the thylakoid membrane is NPQ of excess light energy [120] and the production of membrane antioxidants. NPQ refers to the xanthophyll cycle that dissipates the excessive light energy in the form of heat, thereby reducing ROS formation [120,121], while carotenoids and most of the prenylquinones accumulated in PG are antioxidant molecules playing an important role in photoprotection [122].

Tocopherol is a well-studied membrane antioxidant and its production is strongly increased under high light [123]. Belonging to the tocochromanol family, tocopherol, commonly called vitamin E, refers to four compounds. They differ by the methylation degree of their chromanol ring (α, β, γ and δ). They are lipid-soluble antioxidant molecules [35]. α-Tocopherol (α-T) is the predominant form in photosynthetic tissues, while γ-tocopherol (γ-T) accumulates mainly in seeds. Tocopherols protect membrane lipids against peroxidation and preserve PSII from photoinactivation [123]. Vitamin E-deficient mutants led to the identification of the enzymes involved in tocopherol biosynthesis [21,124]. VTE1 is tocopherol cyclase and catalyzes the cyclisation of 2,3-dimethyl-6-phytyl-1,4-benzoquinone to γ-T [19]. Surprisingly, VTE1 appears to be localized mainly in PG whereas the remainder of the enzymes of the pathway (VTE2, VTE3 and VTE4) was localized at the chloroplast inner envelope membrane [19,36,39,125,126]. The differential localization of VTE1 in PG and the other enzymes at the inner envelope suggest that tocopherol metabolites need to traffic between the compartments [127].

VTE1 is not only implicated in the *de novo* synthesis of tocopherol but also in the recycling of the tocopherol oxidation product α-tocopherol quinol (α-TQH_2) that is produced upon reaction with lipid peroxides. Cyclization by VTE1 completes the conversion of α-TQH_2 into α-T [22,36]. It is interesting to note that reduced quinones are the preferred substrate of VTE1 [36,128].

Interestingly, this regeneration pathway implicates another PG protein, NDC1 (NAD(P)H dehydrogenase 1), which reduces α-tocopherol quinone to α-TQH_2. In the *ndc1* mutant α-tocopherol quinone accumulates to a higher degree than in the WT. NDC1 has a wide substrate specificity and also catalyzes the reduction of plastoquinone (PQ) to plastoquinol contained within PG. In the *ndc1* mutant more PQ is present than in the WT under high light conditions. In higher plants, however, this reaction is inconsequential for bulk photosynthetic electron flow unlike *Chlamydomonas* where NDC1 rather than the NAD(P)H-plastoquinone oxidoreductase complex is responsible for cyclic electron flow around PSI [129]. NDC1 is also required for the efficient production of plastochromanol (PC-8) from PQ that also depends on VTE1. PC-8 is also known to accumulate in PG. PC-8 inhibits lipid peroxidation and is an efficient oxygen scavenger [130,131] primarily produced during senescence rather than under high light [22,132]. Finally and surprisingly NDC1 is also required for the conversion of 2-phytyl-1,4-naphthoquinone, the

immediate precursor of phylloquinone, to phylloquinone in *Arabidopsis* [22,23]. This step requires methylation of the naphthoquinone intermediate by AtMenG [133]. The role of NDC1 in the methylation step is not clear [23]. It is interesting to note, however, that neither *atmenG* nor the *ndc1* mutant have an apparent phenotype and the naphthoquinone intermediate can functionally replace phylloquinone except under high light conditions where oxidative damage of PSI was observed in *atmenG*.

The *Arabidopsis* PG proteomes revealed a surprisingly high number of ABC1-like kinases (ABC1K9-At5g05200, ABC1K1-At4g31390, ABC1K3-At1g79600, ABC1K5-At1g71810, ABC1K6-At3g24190, ABC1K7-At3g 07700) [20,134]. In bacteria and mitochondria, ABC1 homologs regulate ubiquinone synthesis [135,136]. Recently, it has been demonstrated by two independent studies that two PG ABC1-like kinases, ABC1K3 and ABC1K1, regulate prenylquinone metabolism probably through the phosphorylation of VTE1 [26,27]. Martinis et al. characterized the single mutants *abc1k1* and *abc1k3* (Martinis et al. 2013, 2014), while Lundquist et al. concluded that ABC1K1 and ABC1K3 are likely to form a complex and consequently focus their work on the characterization of the double mutant *abc1k1abc1k3* (Lunquist et al., 2013). *abc1k1* and *abc1k1abc1k3* showed sensitivity to light stress, expressed by a rapid leaf bleaching and an inability to accumulate anthocyanin. In the *abc1k1* and *abc1k3* single mutants, VTE1 failed to localize in PG leading to reduced vitamin E (α-T, γ-T and δ-tocopherol) content under high light stress. Surprisingly, however the transcript level of VTE1 was increased. Comparable findings were also made for FBN1a and FBN2 in both single mutants [26,27]. This hints at an underlying post-translational regulation of protein stability. ABC1-like kinase-dependent phosphorylation may stabilize the proteins and/or affect their recruitment to PG. But these results are partially in conflict with those for *abc1k1abc1k3*. A very robust mass spectrometry-based quantification [25] showed that the levels of VTE1, FBN1a and FBN2 are unaffected in isolated PG of *abc1k1abc1k3* compared to WT. In addition, CCD4, ABC1K5 and ABC1K6 levels are strongly downregulated, suggesting that they are stabilized by ABC1K1 and ABC1K3 is required. Moreover, an enrichment of lipoxygenase 3 and 4 (respectively At1g17420 and At1g72520) was observed in isolated PG of *abc1k1abc1k3* under light stress. These two newly identified PG proteins are involved in the synthesis of the plant hormone JA using FA. Recruitment of the JA biosynthetic pathway to PG suggests an increased lipid remobilization for JA production in *abc1k1abc1k3*. An extensive quantification of prenylquinones and carotenoids was also provided, with a specific focus on their distribution and redistribution between PG and thylakoid membrane. Compared to the WT, *abc1k1abc1k3* showed an increased amount of PQ, phylloquinone and β-carotene that are relocated to the PG rather than to the thylakoid membranes. In accordance with a reduced activity of VTE1, they also observed a lower level of PC-8 and α-T that are redistributed toward the thylakoid membranes. Interestingly, a novel quinone (molecular mass of 746.6) was identified in isolated PG of *abc1k1abc1k3*. This putative quinone could be an unknown intermediate of the quinone biosynthesis pathway or an oxidation product of prenyllipids.

The degreening phenotype of *abc1k1abc1k3* is not a strict light stress effect as the same phenotype was obtained under drought stress or nitrogen starvation [25]. The inability of *abc1k1abc1k3* to adapt to elevated light intensity, water or nitrogen deficiency probably leads to excessive singlet oxygen production. The implication of singlet oxygen retrograde signaling in the degreening phenotype is supported by the elevated level of β-cyclocitral found in *abc1k1abc1k3* under light stress [95].

In conclusion, regulation of VTE1 activity by the phosphorylation of ABC1K3 and ABC1K1 is a common finding, which is strongly supported by predictions and experimentation. The PhosPhAt database (The *Arabidopsis* Protein Phosphorylation Site Database, http://phosphat.uni-hohenheim.de/) shows that VTE1 has a phosphorylation hotspot near its N-terminus. Conceivably, phosphorylation at this site affects the activity of the protein. Moreover, VTE1 was shown to be phosphorylated in a phosphoproteome analysis upon nitrogen deficiency [137].

3.2. PG may be involved in the tolerance to the heavy metal cadmium

Heavy metals are either essential (Cu, Zn, Mn) or toxic (Cd, Pb) for plants. Small amounts of essential metals are sufficient to ensure their metabolic role while larger amounts of heavy metals are toxic to the plant (Jasinski et al., 2008). To overcome metal toxicity, phytochelatins, glutathione and ABC transporters are produced in order to sequestrate and/or extrude heavy metals [138,139]. Thus, heavy metal resistance requires the activation of a specific set of genes.

Cadmium (Cd) has a destructive effect on the chloroplast. Upon Cd treatment, the disorganization of the photosynthetic apparatus was observed. More specifically, PSI and associated (light-harvesting) antenna proteins are dismantled while PSII, cytochrome b_6f and ATP-synthase seem less affected [140,141]. Cd can replace other metal ions (Zn, Fe, Ca, Mg) in proteins and may affect the redox state by generating, directly or indirectly, ROS. Regarding lipids, spinach basal leaves showed an increase in xanthophyll content (neoxanthin, violaxanthin and lutein) concomitant with a chlorophyll decrease (chlorosis) in the presence of Cd. The lutein overproduction may represent a defensive mechanism against ROS [141]. Surprisingly, upon Cd treatment, plants seem unable to produce zeaxanthin, a well-described ROS scavenging defense [142, 143]. In the chloroplast, Cd toxicity mimics the symptoms observed during senescence. A notable change is a considerable increase in the number and size of PG [144]. Indeed, Cd-treated *Pisum sativum* plants showed disorganized thylakoid membranes in conjunction with an increase in size and number of PG as well as in the size of starch grains. It was suggested that Cd toxicity induces oxidative stress as in leaf senescence [145].

Interestingly, the expression of the envelope-located ABC1K8 (or OSA1, oxidative-stress related ABC1-like protein) is strongly affected by Cd treatment [146]. To investigate the implication of PG in Cd resistance, effect of Cd on root growth was evaluated for WT, *abc1k1*, *abc1k3* and *abc1k1abc1k3* [25]. However all genotypes showed the same reduced root growth, indicating that ABC1K1 and ABC1K3 are not involved in Cd tolerance. Nevertheless, the PG proteome contains 4 other ABC1-like kinase candidates that have not yet been characterized.

4. Conclusion

In recent years, PG have emerged as an important player in chloroplast lipid metabolism operating as a thylakoid microdomain for metabolite synthesis, repair and disposal. This concerns both the neutral and polar membrane lipids. By their capacities for lipid remodeling at large, PG are implicated in transitions from the chloroplast to the chromoplast and gerontoplast, respectively. While it has been proposed that PG contained in the prolamellar body contribute to thylakoid formation during chloroplast biogenesis, this exciting possibility remains to be demonstrated experimentally. A number of components of PG have been identified, but the full PG lipidome awaits elucidation. In comparison, the PG proteome has been extensively described, but many PG proteins remain to be characterized. In particular the PG ABC1-like kinases remain of great interest as their regulatory functions may extend beyond lipid metabolism and affect a range of other chloroplast pathways [25,26]. The mechanism for protein recruitment to PG is not totally understood. Regarding FBN distribution between PG and thylakoids, Lundquist et al. have suggested that the intrinsic hydrophobicity and isoelectric point of FBN influence their localization to PG [20]. Moreover, it was demonstrated that the nearly entire sequence of FBN7a [147] or FBN1a [148] is required to target recombinant proteins to PG. Lipid remodeling requires molecular communication between PG and thylakoids that with great likelihood implicate their contact sites. The idea of lipid trafficking between the two compartments is supported by the

following observations. (1) PG size is highly dynamic, responding to stress and aging and inversely related to the extent of the thylakoid system. (2) Lipid composition of PG reflects that of the thylakoid. (3) PG enzymes produce lipids that function at the thylakoid. Because a large body of evidence supports this idea, we even postulate bidirectional transport. But the exact mechanism remains mysterious and to be determined in the near future.

Conflict of interest

There is no conflict of interest.

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