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A mechanism implicating plastoglobules in thylakoid disassembly during senescence and nitrogen starvation

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Abstract Plastoglobules are lipid droplets present in all plastid types. In chloroplasts, they are connected to the thylakoid membrane by the outer lipid half-bilayer. The plastoglobule core is composed of neutral lipids most prominently the prenylquinones, triacylglycerols, fatty acid phytyl esters but likely also unknown compounds. During stress and various developmental stages such as senescence, plastoglobule size and number increase due to the accumulation of lipids. However, their role is not limited to lipid storage. Indeed, the characterization of the plastoglobule proteome revealed the presence of enzymes. Importantly it has been demonstrated that these participate in isoprenoid lipid metabolic pathways at the plastoglobule, notably in the metabolism of prenylquinones. Recently, the characterization of two phytyl ester synthases has established a firm metabolic link between PG enzymatic activity and thylakoid disassembly during chloroplast senescence and nitrogen starvation.

Keywords Chloroplast · Fatty acid phytyl ester · Plastoglobule lipid droplets · Prenylquinones · Senescence · Thylakoid membranes

Abbreviations

ABC1	Activity of BC1
AOS	Allene oxide synthase
DGAT	Diacylglycerol acyltransferase
DMPBQ	2,3-Dimethyl-5-phytyl-1,4-benzoquinol
FAPEs	Fatty acids phytyl esters
FBN	Fibrillin
JA	Jasmonate
NDC1	Nicotinamide adenine dinucleotide phosphate (NADPH) dehydrogenase C1
OPDA	12-Oxophytodienoic acid
PG	Plastoglobule
PAP	Plastid-lipid-associated protein
PES	Phytyl ester synthases
PGL	Plastoglobulin
PQH_2	Plastoquinol
ROS	Reactive oxygen species
SAG	Senescence associated gene
TAGs	Triacylglycerols
VTE1	Vitamin E cyclase
WT	Wild type

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Introduction

Plastoglobules (PG) were discovered ~50 years ago by electron microscopy in plant tissues. Present in photosynthetic organisms (Greenwood et al. 1963; Leggett Bailey and Whyborn 1963), PG constitute a specific sub-organellar compartment in various types of plastids such as chloroplasts, chromoplasts and leucoplasts. Easily isolated by floatation on a sucrose gradient, these low density particles contain small amounts of protein, while the interior is filled with neutral lipids, often unsaturated (Vidi et al. 2006; Besagni et al. 2011). For this reason, PG readily react with osmium tetroxide which lead to their “osmiophile” characteristics in electron microscopy.

Structurally related to the oil droplets originating from the endoplasmic reticulum and storing triacylglycerol in their core (Huang 1992), PG arise at the margin of the stromal thylakoid membrane by a "blistering" mechanism and remain contiguous with the outer leaflet of thylakoid lipid bilayer (Austin et al. 2006).

Whereas in green chloroplasts, 95 % of the plastoglobules are single with diameters varying from 45 to 60 nm; in senescing and light stressed chloroplasts, PG tend to be much larger and form interconnected grape-like clusters (Austin et al. 2006). These interconnected PG clusters probably result from secondary blistering events at the surface of an existing PG. The observation of structural connections between the thylakoid membrane and PG and among PG suggests that bidirectional lipid trafficking occurs at the places of contact.

The PG core contains a varying range of neutral lipids including prenylquinones, triacylglycerols (TAGs), fatty acids phytyl esters (FAPEs), carotenoids and others. Plastoquinol (PQH_2) and tocopherol (vitamin E) are the major constituents of the prenylquinone lipid family in plastoglobules, whereas phylloquinone (vitamin K) is also present but in minor concentrations (Lichtenthaler and Peveling 1966; Steinmuller and Tevini 1985; Austin et al. 2006; Lohmann et al. 2006; Gaude et al. 2007; Zbierzak et al. 2010; Lippold et al. 2012).

Conditions provoking oxidative stress such as high light, nitrate starvation, drought, high saline concentration, viral infection, chilling and ozone (Nordby and Yelenosky 1985; Locy et al. 1996; Rey et al. 2000; Oksanen et al. 2001; Gaude et al. 2007; Lichtenthaler 2007) or developmental stages such as senescence and fruit development (Kaup et al. 2002) result in the dismantling of thylakoid membrane. In parallel, the number and the size of lipid droplets increase, due to the accumulation of lipids in their hydrophobic core. However, the precise mechanisms here are not understood.

For many years, the observation that PG accumulate antioxidant molecules as well as catabolic products contributed to the idea that they represent a passive lipid storage site. However, since the characterization of the plastoglobule proteome from *Arabidopsis* chloroplasts and red-pepper chromoplasts, this idea has changed. The proteomics studies revealed the presence of proteins that can be attributed to three different groups: (1) structural proteins called fibrillins (FBN) or plastoglobulins (PGL), (2) enzymes involved in various lipid metabolic pathways and (3) uncharacterized proteins (Vidi et al. 2006; Ytterberg et al. 2006; Lundquist et al. 2012b).

Fibrillins

Fibrillins received their name from fibrils, the carotenoid-containing suborganellar structures of red-pepper chromoplasts that are derived from plastoglobules. The chromoplast fibrils not only contain apolar (carotenoids, tocopherol) and polar (galactolipids, phospholipids) lipids but also a dominant protein of 32 kD protein appropriately termed fibrillin (Deruere et al. 1994). Proteins similar in size were previously identified in the fibrils of Japanese rose (Wuttke 1976), *Nasturtium* (Winkenbach et al. 1976) and *Palisota barteri* (Knoth et al. 1986). The same group of proteins was later termed plastid–lipid associated protein (PAP), because of its localization not only both in chromoplasts but also in chloroplast plastoglobules and its association with lipid-containing structures (Pozueta-Romero et al. 1997). Independently, the fibrillin/PAP protein family localized in plastoglobules was referred to as plastoglobulins (Vidi et al. 2006).

In vitro reconstitution of fibrils by the addition of fibrillin to a mixture of carotenoids and polar lipids experiments suggested a structural function of fibrillin in fibril assembly (Deruere et al. 1994). However, the functions of fibrillins may also directly or indirectly extend to roles in hormonal responses, protection of the photosystem from oxidative stress, resistance to biotic and abiotic stresses or chromoplast pigment accumulation and even a role in lipid transport has been suggested (Singh and McNellis 2011).

Recently, a quantitative proteomics study of PG, identifying 30 proteins, indicated that the FBN are the most abundant PG proteins in *Arabidopsis* leaf rosettes (Lundquist et al. 2012b). Six FBN, including the four major FBN1a, 1b, 2 and 4 (At4g04020, At4g22240, At2g35490, and At3g23400, respectively) accounted for 53 % of the PG protein mass in *Arabidopsis*. Together with six ABC1 (activity of BC1)-like kinases (At5g05200, At4g31390, At1g79600, At1g71810, At3g24190, At3g07700), they make up more than 70 % of the PG protein. In addition, the plastoglobule proteome contains several other major components: CCD4 (carotenoid cleavage dioxygenase-At4g19170), VTE1 (tocopherol cyclase-At4g32770) and NDC1 (NAD(P)H dehydrogenase C1-At5g08740), which account for 3.3, 2.6 and 2.5 % of the protein mass, respectively. PES1 (At1g54570) and PES2 (At3g26840), two phytyl ester synthases represent 2.6 and 1.4 % of the proteome, respectively (Lippold et al. 2012). The remaining proteins account for $<$20 % of the protein mass. These include notably UbiE1 and UbiE2, two methyltransferases (At1g78140 and At2g41040, respectively), M48 protease (At3g27110), as well as a third esterase (At5g41120) related to PES1 and 2, which is described later in this review.

ABC1-like kinases

Among the proteins of the *Arabidopsis* PG proteome, ABC1-like kinases are among the most abundant (Vidi

et al. 2006; Ytterberg et al. 2006; Lundquist et al. 2012b). Even though little is known about functions of the plastoglobule ABC1-like kinases in plants, the prototypical function of this family is the regulation of ubiquinone metabolism in bacteria and mitochondria (Lundquist et al. 2012a). Their mutation leads to accumulation of a ubiquinone precursor and to a shortage of ubiquinone (Cardazzo et al. 1998; Leonard et al. 1998; Poon et al. 2000). In *S. cerevisiae*, *abc1* mutants have a mitochondrial respiratory defect that can be rescued by the addition of exogenous quinones (Bousquet et al. 1991; Brasseur et al. 1997). Moreover, ABC1 is able to complement the *coq8* mutant that is defective in ubiquinone synthesis (Do et al. 2001). In bacteria, the ubiB gene homolog of *ABC1* is required for the first monooxygenase step in ubiquinone synthesis (Poon et al. 2000). In humans, mutation of an ABC1-like homolog leads to neuromuscular defects such as ataxia (Mollet et al. 2008). Recently, two putative kinases have been characterized in *Arabidopsis*: AtOSA1 (*Arabidopsis* oxidative stress related ABC1-like protein, At5g64940) localized at the chloroplast inner envelope membrane (Jasinski et al. 2008) and AtACDO1 (ABC1-like kinase related to chlorophyll degradation and oxidative stress, At4g31390) (Yang et al. 2012). Both were linked to oxidative stress under high light conditions. Thus, the chloroplast localization together with the phenotypes and the analogy to mitochondrial and bacterial ABC1-like kinases suggests that plastoglobule ABC1-like kinase proteins regulate prenylquinone metabolism and that they may do so via phosphorylation of enzymes in the pathway (Ytterberg et al. 2006; Lundquist et al. 2012a). Indeed, in yeast, it has been demonstrated that ABC1/Coq8 is required for the phosphorylation of Coq3, Coq5 and Coq7, three enzymes involved in ubiquinone pathway. Moreover, the human Coq8 ortholog ADCK3 rescues the phosphorylation of several Coq proteins in the yeast *coq8* mutant as well as the defective phenotype of the strain (Xie et al. 2011).

The following group of proteins, with regard to their abundance in the PG proteome, includes CCD4, which has been implicated in carotenoid degradation (Ahrazem et al. 2010). Further indications for the implication of plastoglobules in carotenoid metabolism stems come from the identification of z-carotene desaturase (ZDS), lycopene b-cyclase (LYC-b or CYC-b), and two b-carotene b-hydroxylases (CrtR-b) in the PG chromoplast proteome of tomato (Ytterberg et al. 2006) (Fig. 1c).

Implication of plastoglobules in isoprenoid synthesis

Immunoelectron tomographic studies performed on two *Arabidopsis* PG proteins, the plastoglobulin PGL35 (At4g04020) and the tocopherol cyclase VTE1 revealed that PG proteins are located at the surface of the PG, presumably in contact with the head group of the polar lipids (Austin et al. 2006). VTE1, even penetrated the polar lipid monolayer, and extended into the core of the PG potentially allowing it to access its hydrophobic substrates (Austin et al. 2006; Kobayashi and DellaPenna 2008; Mene-Saffrane et al. 2010).

The role of PG in isoprenoid metabolism is highlighted by two PG enzymes (Piller et al. 2012) (Fig. 1a): VTE1 and NDC1. Under oxidative stress, prenylquinone synthesis represents an important plant response to protect the thylakoid membranes against reactive oxygen species (ROS) (Gruszka et al. 2008). PG have now been implicated in the synthesis, storage and regeneration of these antioxidant molecules that include tocopherols, plastoquinone, plastochromanol and phylloquinone. PG may release and exchange prenylquinones with the thylakoid membranes through the attachment sites (Austin et al. 2006).

Tocopherol accumulates in plastoglobules under high light stress (Havaux et al. 2005; Brehelin et al. 2007) and may in part explain the enlargement of PG under such conditions. VTE1 mediates the conversion of 2,3-dimethyl-5-phytyl-1,4-benzoquinol (DMPBQ) into γ-tocopherol. While most of the VTE1 appears to be present in PG, the three enzymes (VTE2, 3, 4) catalyzing the other biosynthetic reactions have been localized to the chloroplast inner envelope membrane (Soll et al. 1985; Cheng et al. 2003; Vidi et al. 2006; Kobayashi and DellaPenna 2008; Zbierzak et al. 2010). VTE1 has also been implicated in the recycling of the tocopherol oxidation product, α-tocopherol quinone, that accumulates in response to oxidative stress (Kobayashi and DellaPenna 2008).

The NADPH dehydrogenase NDC1, localized at plastoglobules, has been implicated in the re-oxidation of the reduced pool of plastoquinol (PQH_2-9) accumulated in plastoglobules during high light stress (Szymanska and Kruk 2010; Zbierzak et al. 2010; Piller et al. 2011). This pool can also serve as a substrate for VTE1 to produce plastochromanol-8 (Mene-Saffrane et al. 2010; Szymanska and Kruk 2010; Zbierzak et al. 2010). A role of NDC1 in the biosynthesis of phylloquinone has also been demonstrated using a non-targeted lipidomic analysis of the *ndc1* mutant. The *ndc1* mutant completely lacked phylloquinone but accumulated its immediate precursor, demethyl-phylloquinone instead (Piller et al. 2011). The enzyme AtMENG, which catalyzes this methylation, had been identified previously (Lohmann et al. 2006) and was normally expressed in the *ndc1* mutant. The mechanism of NDC1 in phylloquinone synthesis currently remains unknown.

Role of plastoglobules during leaf senescence

The implication of PG in the storage of catabolic molecules produced during senescence and nitrogen

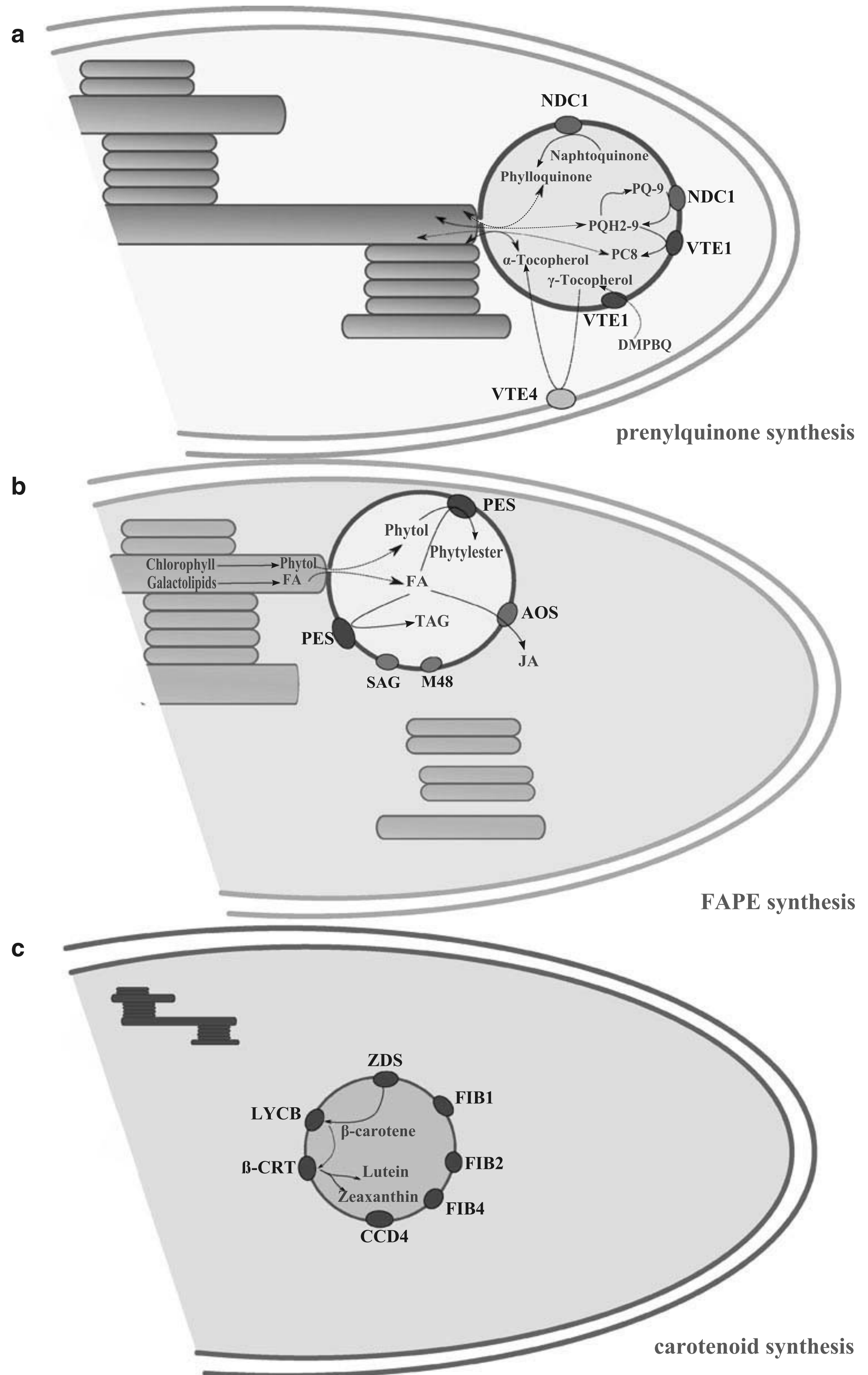

Fig. 1 Implication of plastoglobule enzymes in lipid metabolic pathways. **a** During high light stress, NDC1 and VTE1 are implicated in the synthesis and the recycling of prenylquinones (phylloquinones, plastoquinol (PQH_2-9), plastochromanol (PC8), tocopherol). **b** During senescence, PES1 and PES2 synthesize fatty acid phytyl esters (FAPEs) from fatty acids (FA) and phytol, leading to plastoglobule enlargement and disassembly of the thylakoid membrane. **c** PG-localized enzymes are involved in carotenoid synthesis principally in chromoplasts

starvation has been described previously (Kaup et al. 2002; Gaude et al. 2007). However, the characterization of two PG acyl transferases has led to an advance in mechanistic understanding of the accumulation of fatty acid phytyl esters and triacylglycerols (Fig. 1b).

Senescence is essentially a highly coordinated cascade of events leading to cell death. During this process, leaf

cells undergo series of changes in gene expression, metabolism and structure, which lead to a decline in photosynthetic activity and loss of chlorophyll. However, variations in the symptoms of senescence exist and leaves may remain green longer than normal in certain mutants. Functional *stay-greens* genotypes or mutants retain both chlorophyll and photosynthetic competence, whereas cosmetic *stay-greens* retain their chlorophyll but loose photosynthetic activity (Hortensteiner 2009).

Senescence is controlled by environmental and autonomous factors (Gan and Amasino 1997; Quirino et al. 2000). Internal factors include age, phytohormones and reproductive development, whereas external cues include stress conditions such as nutrient deficiency, pathogen infection, drought, low and high temperature and shading. Thus, the expression patterns of numerous genes are common between such stress responses and leaf senescence (Lim et al. 2007).

Hormones provide a means for plants to signal and control leaf senescence. Indeed, cytokinines, ethylene, auxin, jasmonic acid (JA), abscisic acid and salicylic acid are key plant hormones mediating senescence in different ways. Stresses which affect the synthesis of these phytohormones or their exogenous application lead to a modification in gene expression underlying senescence (Weaver et al. 1998). For example, cytokinines delay senescence by a negative regulation of the promoter of the senescence-associated gene SAG12 (Gan and Amasino 1995). In contrary, exogenously applied ethylene induces premature leaf senescence in *Arabidopsis*, and ethylene insensitive mutants *etr1-1* and *ein2/ore3* showed increased leaf longevity (Guiboileau et al. 2010). Similarly, methyl jasmonate as well as its precursor JA lead to an increased expression of SAG genes including SAG14, SEN4, SEN5 and rVPE. Moreover, the JA-insensitive mutant *coi1* (coronatine insensitive 1) is defective in JA-dependant senescence (He et al. 2002).

A large set of regulatory SAGs has been isolated from various plant species. Up-regulated during senescence, they trigger leaf senescence by signal perception and transduction such as receptor-like kinases and transcription factors (Quirino et al. 2000; Lim et al. 2007). Among at least 800 SAGs identified in *Arabidopsis*, 20 different families of transcription factors are significantly overexpressed during senescence. The largest groups being NAC, WRKY, C_2H_2-type zing finger and MYB proteins (Hinderhofer and Zentgraf 2001; Guo and Gan 2006). These transcription factors control the expression of many genes of senescence (Buchanan-Wollaston and Ainsworth 1997; Lin and Wu 2004). Among them, WRKY53 was shown to play a central role in early leaf-senescence and has various targets such as SAGs, PR genes, and additional WRKY factors. Indeed, the *wrky53* mutant manifested a delay in senescence whereas overexpression caused precocious senescence (Miao et al. 2004).

During senescence, many of the SAGs regulate the catabolic events such as protein, lipid and nucleic acid degradation that ultimately lead to cell death (Thompson et al. 1998). However, this programmed cell death also coincides with a remobilization of nutrients (e.g. nitrogen and phosphorus) that are relocated from the senescing tissue to the developing seeds (Quirino et al. 2000). Thus, leaf senescence also has aspects of a recycling process important for re-production and plant fitness.

Probably the first visible manifestations of leaf senescence occur at the level of the chloroplast. The dismantling of the thylakoid membrane, which consists to 80 % of galactolipids, results in the release of free fatty acids. In parallel, chlorophyll is catabolized and free phytol is released by the pheophytin pheophorbide hydrolase (PPH) (Harris and Arnott 1973; Hortensteiner 2006). These hydrolytic products are considered toxic and can be metabolized to form fatty acid phytyl esters, tocopherols and triacylglycerols. All three of these accumulate in plastoglobules during senescence and nitrogen starvation (Kaup et al. 2002; Ischebeck et al. 2006; Vidi et al. 2006; Gaude et al. 2007).

The chlorophyll catabolic pathway (Hortensteiner 2006) as well as the incorporation of phytol into tocopherol via phosphorylation by the phytol kinase VTE5 (Valentin et al. 2006) are well documented. However, the conversion mechanism of phytol to fatty acid phytyl esters was unclear until the recent characterization of two PG enzymes, phytyl ester synthase, PES1 and PES2. These two acyltransferase proteins, up-regulated during senescence and nitrogen starvation, belong to the ELT (esterase/lipase/thioesterase) family that includes six members in *Arabidopsis*. They can esterify either diacylglycerol or the phytol (stemming from degradation of the thylakoid membrane and the chlorophyll) with free fatty acids, resulting in the formation of triacylglycerols and FAPEs, respectively (Lippold et al. 2012). According to the nature of the acyl groups in phytyl ester and triacylglycerols, fatty acids may be derived from de novo synthesis or galactolipid degradation which provides a pool enriched in fatty acids C16:3 and C18:3 (Browse et al. 1986).

In the *pes1–pes2* double mutant under nitrogen starvation, the amount of FAPEs was reduced by 85 % suggesting that these two enzymes play a major role (Lippold et al. 2012). In contrary, the amount of TAGs in the mutant leaves is only 30 % lower, certainly due to the implication of other diacylglycerol acyltransferase enzymes such as DGAT1 (Zou et al. 1999; Dahlqvist et al. 2000; Lardizabal et al. 2001; Kaup et al. 2002). Moreover, in the updated and quantitative plastoglobule proteome study published recently by Lundquist and colleagues, in addition to PES1

and 2, a third esterase (At5g41120) was identified that may also participate in the synthesis of FAPEs and TAGs in plastoglobules (Lundquist et al. 2012b).

Phenotypically, a delay in senescence, but not a stay green phenotype, is observed in *pes1–pes2* double mutant. It is characterized by the persistence of a pale green phenotype for a longer period of time than in the wild type. This pale green phenotype correlates with the retention of a thylakoid membrane network, which could explain the presence of residual chlorophyll (Lippold et al. 2012). The presence of a predicted hydrolase domain of the α/β-superfamily in PES1 and 2 proteins in addition to the esterase domain suggests that these two proteins may also have lipase activity and be directly involved in galactolipid catabolism.

Fatty acids C18:3 and C16:3 originate from galactolipid degradation and are precursors for jasmonate biosynthesis (Gfeller et al. 2010). This phytohormone is not only involved in stress resistance (e.g. wounding, pathogen attack) but also in senescence induction (Shan et al. 2011). Allene oxide synthase (AOS), a key enzyme in jasmonate biosynthesis was originally identified in two proteome studies (Vidi et al. 2006; Ytterberg et al. 2006), but had already been reported to associate with chloroplast inner envelope membrane in tomato (Froehlich et al. 2001) and in *Arabidopsis* (Ferro et al. 2010). Thus, despite the clear presence of AOS in plastoglobules, it can not consider a *bona fide* plastoglobule protein due its presence in other chloroplast membrane compartments (Lundquist et al. 2012a). Nevertheless, the presence of AOS in plastoglobules, which catalyzes the formation of jasmonate precursor 12-oxophytodienoic acid (OPDA), suggests that plastoglobules may also participate in the production of jasmonate during senescence.

Moreover, under high light and low temperature, it has been demonstrated that the production of jasmonate is linked to the presence of fibrillins in PG. Indeed, the addition of jasmonate rescues the mutant phenotype of *fib1–2* RNAi plants, characterized by a retarded shoot growth, a deficit in anthocyanin accumulation and increased oxidative stress symptoms. In addition, the number of PG is lower in *fib1–2* RNAi plants than in wild type plants, concomitant with a reduction of TAGs in PG that makes less C18:3 fatty acid, the precursor of jasmonate, available in plastids. Indeed, the fibrillins in PG could influence early steps of jasmonate synthesis during stress (Youssef et al. 2010).

Finally, the identification of an unknown SAG protein in the plastoglobule proteome and a M48 protease in the same co-expression network as PES1 and 2 (termed "senescence module" by Lundquist et al.) firmly anchors plastoglobules in the senescence program (Lundquist et al. 2012b).

Conclusions

PG are lipoprotein particles that serve as an active lipid reservoir during stresses such as high light or during senescence. The characterization of the PG proteome revealed the presence of enzymes that provide PG with enzymatic capability for lipid synthesis (Vidi et al. 2006; Ytterberg et al. 2006; Lundquist et al. 2012b).

The *Arabidopsis* PG proteome contains three groups of proteins: fibrillins, enzymes involved in various lipid metabolic pathways and uncharacterized proteins. Among these PG proteins, fibrillins and ABC-like kinases constitute the most abundant families (Lundquist et al. 2012b).

In particular, the important role of PG proteins in prenylquinone metabolism is now well established notably via the characterization of NDC1 and the discovery of new aspects of VTE1 function (Vidi et al. 2006; Szymanska and Kruk 2010; Zbierzak et al. 2010; Piller et al. 2011, 2012).

Furthermore, the concomitant presence of proteins overexpressed during senescence (e.g. SAG, PES1/-2, M48) and implicated in jasmonate synthesis (AOS, FIB1-2) as well as co-expression networks strongly support a role for PG in the senescence process (Lundquist et al. 2012b). The mechanisms by which these actors influence senescence are still far from fully known. However, it is has now become clear that the PES1 and PES2 activities through their role in the biosynthesis of fatty acid phytyl esters and triacylglycerols are major contributors to thylakoid disassembly and plastoglobule enlargement during senescence and nitrogen deprivation.

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Conflict of interest The authors declare that they have no conflict of interest.

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