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DARWIN REVIEW

Structure and dynamics of thylakoids in land plants

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Abstract

Thylakoids of land plants have a bipartite structure, consisting of cylindrical grana stacks, made of membranous discs piled one on top of the other, and stroma lamellae which are helically wound around the cylinders. Protein complexes predominantly located in the stroma lamellae and grana end membranes are either bulky [photosystem I (PSI) and the chloroplast ATP synthase (cpATPase)] or are involved in cyclic electron flow [the NAD(P)H dehydrogenase (NDH) and PGRL1–PGR5 heterodimers], whereas photosystem II (PSII) and its light-harvesting complex (LHCII) are found in the appressed membranes of the granum. Stacking of grana is thought to be due to adhesion between Lhcb proteins (LHCII or CP26) located in opposed thylakoid membranes. The grana margins contain oligomers of CURT1 proteins, which appear to control the size and number of grana discs in a dosage- and phosphorylation-dependent manner. Depending on light conditions, thylakoid membranes undergo dynamic structural changes that involve alterations in granum diameter and height, vertical unstacking of grana, and swelling of the thylakoid lumen. This plasticity is realized predominantly by reorganization of the supramolecular structure of protein complexes within grana stacks and by changes in multiprotein complex composition between appressed and non-appressed membrane domains. Reversible phosphorylation of LHC proteins (LHCPs) and PSII components appears to initiate most of the underlying regulatory mechanisms. An update on the roles of lipids, proteins, and protein complexes, as well as possible trafficking mechanisms, during thylakoid biogenesis and the de-etiolation process complements this review.

Key words: Bifurcation, CURT1, electron microscopy, grana, lateral heterogeneity, margins, phosphorylation, steric hindrance, STN7, STN8, thylakoid, VIPP1.

Introduction

Thylakoids are the internal membranes of chloroplasts and cyanobacteria, and provide the platform for the light reactions of photosynthesis. Chloroplasts of land plants contain grana, characteristic cylindrical stacks of membrane discs with a typical diameter of 400 nm that comprise ~5–20 layers of thylakoid membrane (Mustárdy and Garab, 2003; Mullineaux, 2005). A single granum consists of a central core of appressed membranes, capped by stroma-exposed 'grana end membranes' at the top and bottom, and the tightly curved margins that form the periphery of each discoid sac (Albertsson, 2001). Grana stacks are interconnected by stroma-exposed membrane pairs of up to few micrometres in length, the so-called 'stroma lamellae'. All thylakoid membranes within a chloroplast form a continuous network that encloses a single lumenal space (Shimoni *et al.*, 2005).

Although many biological textbooks include illustrations of the unique and intriguing architecture of thylakoids in land plants, relatively little is known about how it is generated, maintained, and modified. This review summarizes the present state of research on the structure, lateral heterogeneity, dynamics, and biogenesis of thylakoids from land plants and highlights new developments in these fields.

Architecture of thylakoids in land plants

A structural hallmark of thylakoid membranes in plants is their stacking to form the so-called grana thylakoids, which are interconnected by an unstacked but continuous network of stroma lamellae. Grana cylinders are made up of stacks of flat grana membrane discs with a diameter of ~300–600 nm, which are enwrapped in the stroma lamellae. In dark-adapted grana from *Arabidopsis thaliana*, membrane bilayers are (on average) 4.0 nm thick, lumen thickness is 4.7 nm, and discs are separated by a 3.6 nm gap (Kirchhoff *et al.*, 2011).

The exact three-dimensional architecture of grana is still under debate, and two quite different interpretations of the wealth of electron microscopy data obtained during the past several decades have been proposed: the 'helical model' and various 'fork/bifurcation models' (reviewed in Daum and Kühlbrandt, 2011; Kirchhoff, 2013*a*). In the helical model (Paolillo, 1970), thylakoids comprise a fretwork of stroma lamellae, which wind around grana stacks as a right-handed helix, connecting individual grana discs via narrow membrane protrusions (Fig. 1). In its latest form (Mustárdy and Garab, 2003; Mustárdy *et al.*, 2008), the model suggests a bipartite structure consisting of a cylindrical granum body, made of discs piled on top of each other, around which the stroma lamellae are wound as right-handed helices. The grana are connected to each other solely via the stroma lamella helices, which are tilted at an angle of between 10 ° and 25 ° with respect to the grana stacks (Mustárdy *et al.*, 2008; Daum *et al.*, 2010; Austin and Staehelin, 2011) and make multiple contacts with successive layers in the grana through slits located in the rims of the stacked discs.

The most significant difference between this helical model and competing alternative models is that the latter postulate that the grana themselves are formed by bifurcations of stroma lamellae. Thus Arvidsson and Sundby (1999) suggested that a granum is composed of piles of repeat units, each containing three grana discs, which are formed by symmetrical invaginations of a thylakoid pair caused by bifurcation of the thylakoid membrane (Fig. 1). With this model it is easier to account for the unstacking and restacking of grana under changing light conditions. More recently, Shimoni *et al.* (2005) presented another model, in which grana discs are paired units formed by simple bifurcation of stroma thylakoids (Fig. 1). Here, the granum–stroma assembly is formed by bifurcations of the stroma lamellar membranes into multiple parallel discs. The stromal membranes form wide, slightly undulating, lamellar sheets that intersect the granum body roughly perpendicular to the long axis of the granum cylinder. Instead of winding around the grana and fusing to form multiple granum layers at various levels (as in the helical model), each stroma lamellar sheet enters and exits the granum body in approximately the same plane (Shimoni *et al.*, 2005; Brumfeld *et al.*, 2008). Adjacent granum layers are joined not only through the stroma lamellae (as proposed by the helical model), but also via the bifurcations and through direct membrane bridges. The latter are formed by bending of the granum discs, leading to fusion with their neighbours at the edges (Fig. 1). This model has also been used to explain the rearrangements in thylakoids during state transitions (Chuartzman *et al.*, 2008).

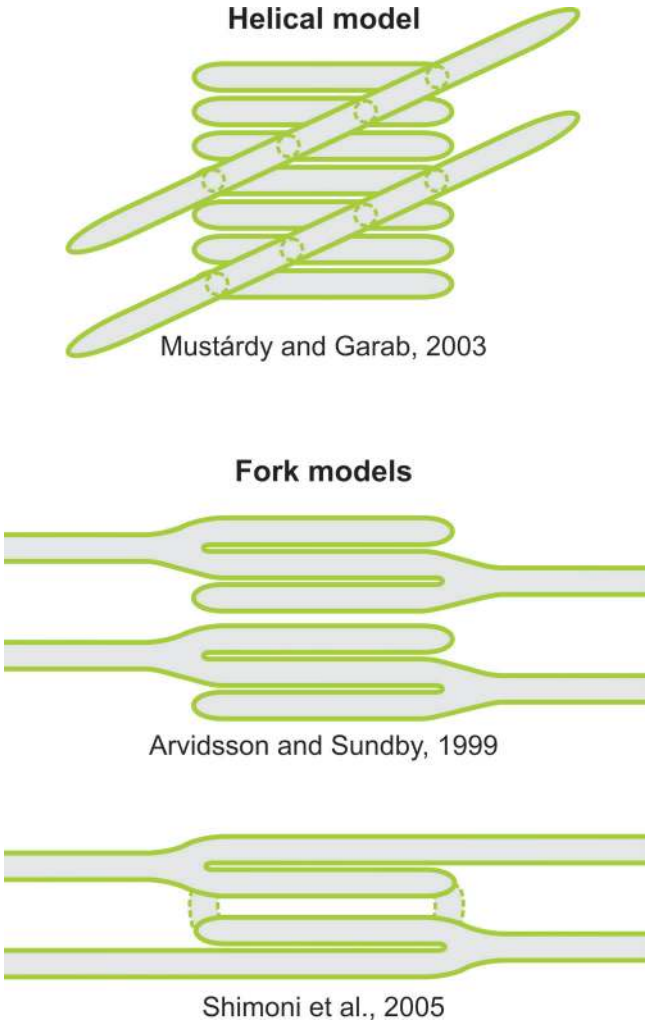

Fig. 1. Models of thylakoid architecture. In the helical model (reviewed in Mustárdy and Garab, 2003; Mustárdy *et al.*, 2008), a fretwork of stroma lamellae, which wind around the grana stacks as a right-handed helix, connects to individual grana discs via narrow membrane protrusions (indicated by dotted circles). In fork models, stroma lamellae bifurcate to generate grana discs. Arvidsson and Sundby (1999) suggest that grana consist of repetitive units, each containing three grana discs formed by symmetrical invaginations of a thylakoid pair. Later, Shimoni *et al.* (2005) proposed that grana are composed of paired layers formed by bifurcations of stroma lamellar sheets. These layers are interconnected by membrane bridges (dotted lines) that emerge from one layer and fuse to the next.

The mutual incompatibility of the helical and bifurcation models has led to much debate (Brumfeld *et al.*, 2008; Mustárdy *et al.*, 2008; Austin and Staehelin, 2011; Daum and Kühlbrandt, 2011; Nevo *et al.*, 2012). However, recent tomographic data presented by Daum *et al.* (2010) and Austin and Staehelin (2011) clearly support the helical model, prompting Daum and Kühlbrandt (2011) to conclude: 'Taken together, the tomographic data presented by Daum *et al.* (2010), Austin and Staehelin (2011), and here show that the helix model of thylakoid membrane architecture in chloroplasts is correct. Other models do not describe this architecture correctly and can now be safely discarded.'

Nevertheless, the controversy persists, as Nevo *et al.* (2012) point out that '…forks or bifurcations were observed and noted in the works of Daum *et al.* (2010), as well as by Austin and Staehelin (2011; here, termed 'branches' or 'junctional connections').'

Characteristics of lateral heterogeneity

The various photosynthetic complexes differ not only in abundance (Table 1) but also in their distribution within the photosynthetic membranes. The term 'lateral heterogeneity' refers to the observation that stroma lamellae and grana differ in their protein composition (Andersson and Anderson, 1980; Albertsson, 2001; Dekker and Boekema, 2005; Nevo *et al.*, 2012; Kirchhoff, 2013*a*), with photosystem II (PSII) and light-harvesting complex II (LHCII) being concentrated in the grana—where they can form super- and megacomplexes and semi-crystalline arrays—while photosystem I (PSI) with its light-harvesting complex (LHCI) and the chloroplast ATP synthase (cpATPase) are localized in the unstacked thylakoid regions; that is, the stroma lamellae and grana end membranes (Fig. 2). The allocation of the cytochrome b_6f complex (Cyt b_6f) between grana and stroma lamellae is less clear, but it is generally assumed that the complex can be found in both appressed and non-appressed regions of thylakoids (reviewed in Dekker and Boekema, 2005; Nevo *et al.*, 2012). The thylakoid complexes involved in cyclic electron flow, the NDH complex and the PGRL1–PGR5 heterodimer, are less abundant than these four major thylakoid complexes (see Table 1) and are located in the stroma lamellae (Lennon *et al.*, 2003; Rumeau *et al.*, 2007; Hertle *et al.*, 2013) where they can functionally interact with PSI as electron donor. The bulk of the NDH complex protrudes into the stroma, which precludes its location in grana, but PGRL1 homodimers have been detected in grana (Hertle *et al.*, 2013) (Fig. 2).

Although several of the major thylakoid multiprotein complexes have been detected in margin-enriched fractions of thylakoids, these signals most probably reflect contamination by regions adjacent to grana margins (Dekker and Boekema, 2005). The marked curvature of thylakoid membranes at the grana margins would be expected to exclude larger multiprotein complexes and, indeed, grana margins have been thought to be essentially protein free (reviewed in Dekker and Boekema, 2005). However, following the recent demonstration, by immunogold labelling, that the CURT1 proteins—small polypeptides with two transmembrane regions, a tentative N-terminal amphipathic helix, and a relatively low mol. wt (11.0–15.7 kDa)—are localized to grana margins (Armbruster *et al.*, 2013), this view must be revised. In this instance, envelope-free chloroplasts of wild-type plants and plants containing tagged CURT1A variants were first immunodecorated with specific antibodies and then treated with the appropriate gold-labelled secondary antibodies, which were subsequently visualized by scanning electron microscopy. Because this approach directly localizes proteins without fractionation or extensive fixation steps, it is ideally suited for the detection of proteins located in defined subcompartments within stroma-exposed areas of thylakoids (like margins). Interestingly, the CURT1 proteins appear to control the level of grana stacking, which points to an unsuspected role for grana margins in regulating the extent of thylakoid membranes in the appressed regions present in grana (see below).

Table 1. *Summary of the total and relative abundances of photosynthetic complexes*

In the estimate of the abundance of NDH based on quantification of PsaA (which detects PSI–NDH supercomplexes), the value reported in the literature was halved, because it is likely that each NDH complex is associated with two PSI complexes (Peng et al., 2008). The value for CURT1 was obtained by summing the values reported for CURT1A, B, and C (Armbruster et al., 2013). To calculate the abundance of protein complexes relative to PSI, values obtained from Kirchhoff et al. (2002) are expressed with respect to the value for PSI obtained in that study, whereas values from Hertle et al. (2013) and Armbruster et al. (2013) were related to the value for PSI obtained in Armbruster et al. (2013). For quantitation of the NDH complex based on measurements of NdhI and PsaA, the relative values (with respect to PSI) calculated in the corresponding publications were used.

Component	Concentration (mmol/mol of Chl)	Method	% of PSI	Reference
PSII	2.99 ± 0.22	Absorbance changes	133	Kirchhoff *et al.* (2002)
LHCII	33.68 ± 0.76	Coomassie staining	1497	Kirchhoff *et al.* (2002)
PSI	2.25 ± 0.17	Absorbance changes	100	Kirchhoff *et al.* (2002)
	2.16 ± 0.24	PsaD immunoblot	100	Armbruster *et al.* (2013)
Cyt b_6f	1.29 ± 0.05	Absorbance changes	57	Kirchhoff *et al.* (2002)
	1.35 ± 0.005	PetD immunoblot	63	Hertle *et al.* (2013)
cpATPase	0.95 ± 0.06	Coomassie staining	42	Kirchhoff *et al.* (2002)
NDH	0.09 ± 0.005	NdhD immunoblot	4	Hertle *et al.* (2013)
		NdhI immunoblot	1–2	Burrows *et al.* (1998)
		PsaA immunoblot	0.25–2.5	Peng *et al.* (2008)
PGRL1	0.70 ± 0.003	Immunoblot	32	Hertle *et al.* (2013)
PGR5	0.09 ± 0.005	Immunoblot	4	Hertle *et al.* (2013)
CURT1	0.41	Immunoblot	19	Armbruster *et al.* (2013)

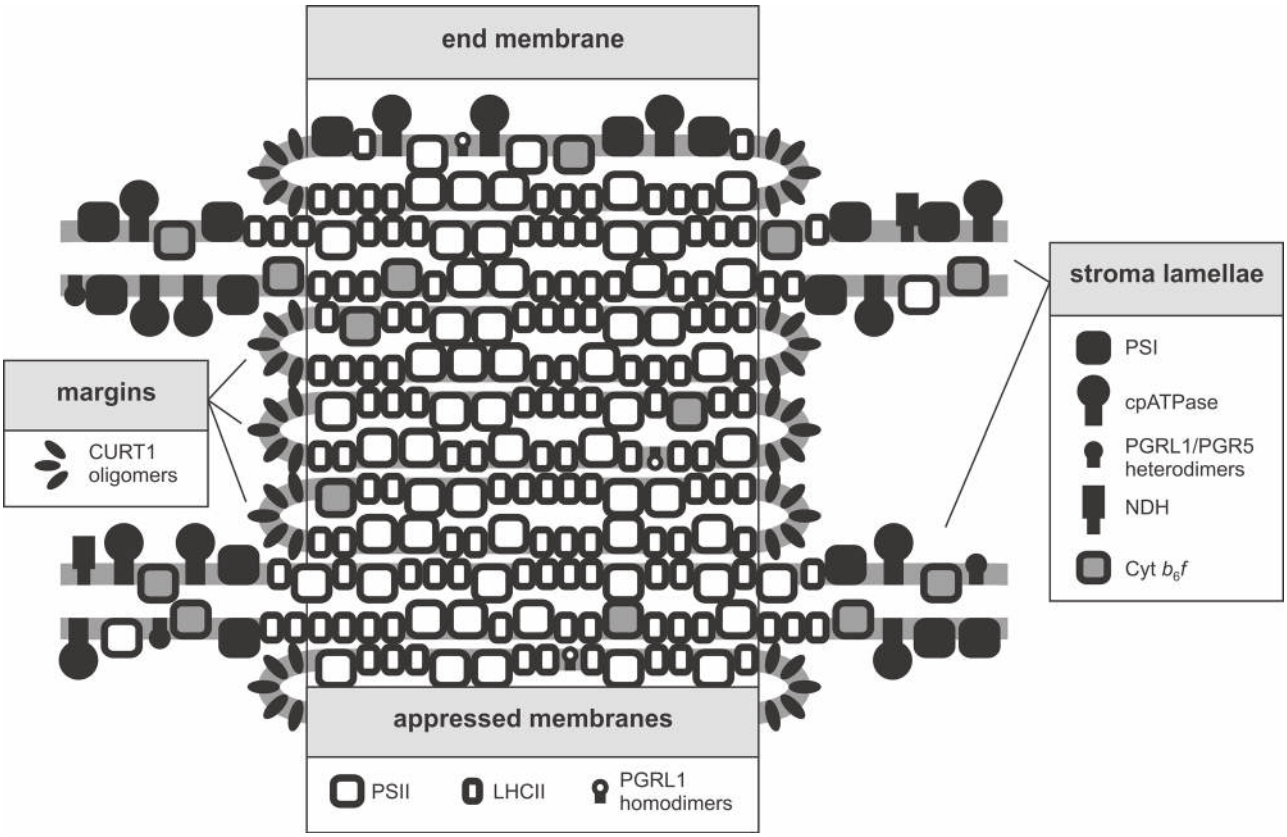

Fig. 2. Lateral heterogeneity in thylakoids. Protein complexes predominantly located in the stroma lamellae [PSI, cpATPase, NAD(P)H dehydrogenase (NDH), and PGRL1–PGR5 heterodimers] are shaded in black, whereas complexes found in the appressed membranes of the grana (PSII dimers and LHCII trimers) are shown in white. The dimeric Cyt b_6f complex (grey shading) is thought to be present in both stroma lamellae and appressed regions (Dekker and Boekema, 2005), whereas CURT1 oligomers (black ellipses) are found in the highly curved regions of thylakoids, in particular in the grana margins (Armbruster *et al.*, 2013). Monomeric PSII complexes that are undergoing repair can be also found in the stroma lamellae (Baena-González and Aro, 2002). NDH and PGRL1–PGR5 heterodimers can form complexes with PSI in stroma lamellae (DalCorso *et al.*, 2008; Peng *et al.*, 2008). Note that, in general terms, the protein composition of the end membranes is assumed to be similar to that of stroma lamellae. Tomography and immunogold labelling experiments have confirmed that the cpATPase and Cyt b_6f complexes, respectively, are located in end membranes (Daum *et al.*, 2010; Armbruster *et al.*, 2013). Moreover, PGRL1 homodimers are enriched in the grana and might represent a pool of physiologically inactive PGRL1 molecules that can be recruited under certain conditions (for instance high light) (Hertle *et al.*, 2013). Note that the relative levels of the different multiprotein complexes depicted in the figure do not reproduce the values given in Table 1.

Mechanisms leading to membrane stacking and lateral heterogeneity

The physicochemical forces that control membrane stacking and the lateral segregation of protein complexes are not completely understood. Because of the extremely high density of photosynthetic protein complexes in the thylakoid membranes, membrane appression and lateral segregation of the photosynthetic protein complexes are thought to be intimately linked (Nevo *et al.*, 2012). The unequal distribution of PSI and the cpATPase can be explained in a straightforward way as the bulk of their mass protrudes from the membrane. Therefore, steric hindrance excludes both complexes from grana stacks. Conversely, both LHCII and PSII have relatively flat stromal surfaces that allow them to be accommodated in tightly stacked grana thylakoids, where the distance between neighbouring membranes is only a few nanometres (reviewed in Kirchhoff, 2013*a*). Thus, a scenario can be imagined in which the tendency of PSII–LHCII complexes to self-associate into densely packed macrodomains promotes their segregation to certain loci, where they act as 'staples' to give rise to appressed regions and ensure the exclusion of the sterically incompatible cpATPase and PSI complexes.

Why and how are thylakoid membranes attracted to each other such that they form grana? Most research on this topic has been guided by the maxim: 'If LHCII is the major protein component of grana, then grana formation must be mediated by LHCII.' Indeed, that LHCII polypeptides mediate membrane appression *in vitro* and thylakoid stacking *in vivo* was proposed 30 years ago (Day *et al.*, 1984). Subsequently, based on an analysis of the charge distribution on LHCII trimers exposed on the stromal surface, it was suggested that non-specific interaction of positively charged N-terminal peptides on one membrane with a negatively charged surface patch on trimers protruding from the opposite membrane is likely to play a major role in the cohesion of thylakoid grana (Standfuss *et al.*, 2005). Moreover, constitutive expression of pea Lhcb1 in transgenic tobacco plants indeed leads to increased grana stacking (Labate *et al.*, 2004), and overexpression of certain LhcbM proteins in *Chlamydomonas* is associated with more tightly appressed membrane regions (Mussgnug *et al.*, 2005), indicating that increased concentrations of LHCII result in

more stacking. This 'velcro-like', non-specific interaction of LHCII trimers in apposed thylakoid membranes (Standfuss *et al.*, 2005) would also explain why phosphorylation of LHCII, which effectively neutralizes one positive charge at the N-terminus, weakens this interaction, making it easier for LHCII to relocate towards PSI during state transitions (reviewed in Pesaresi *et al.*, 2011). By analogy with this, it has been suggested that the deficit in PSII phosphorylation in the thylakoid kinase mutant *stn8* increases the diameter of grana discs by reducing membrane repulsion due to negatively charged phosphate groups, thus enhancing cohesion between thylakoid membranes of adjacent discs (Fristedt *et al.*, 2009).

However, several observations clearly indicate that the notion that LHCII alone mediates the cohesion of thylakoid membranes in the grana is too simplistic. (i) *Arabidopsis* mutants that are virtually devoid of LHCII trimers show no defects in grana formation, and their thylakoid architecture is essentially normal (Andersson *et al.*, 2003). In fact, in these LHCII-less plants, the role of LHCII is taken over by a normally minor and monomeric complex, CP26, which is synthesized in large amounts and organized into trimers (Ruban *et al.*, 2003). This at least permits the conclusion that grana stacking does not strictly require LHCII. Instead, it seems likely that CP26 can, in principle, substitute for the putative function of LHCII in grana stacking. (ii) Mutants that lack PSII but still accumulate LHCII, such as *hcf136*, show markedly enlarged grana that extend almost throughout the chloroplast and display reduced spacing (Meurer *et al.*, 1998), indicating that grana formation is not strictly dependent on the formation of PSII–LHCII aggregates either. However, under variable irradiances, dynamic changes in the stacking of wild-type thylakoid membranes depend on the reversible reorganization of LHCII–PSII supracomplexes and therefore the LHCII/PSII ratio present in grana stacks (Anderson *et al.*, 2012). (iii) More recently, data were presented which suggest that factors located in the grana margins can regulate the dimensions of grana stacks. Thus the level of CURT1 proteins localized to margins appears to control the dimensions of grana stacks without changing the amounts of LHCII and PSII accumulated, such that plants without CURT1 proteins display grana with a significantly increased diameter but far fewer layers of membrane, which form 'pseudograna' without margins (Armbruster *et al.*, 2013) (Fig. 3). This phenotype resembles that of *stn8* thylakoids without PSII core phosphorylation (Fristedt *et al.*, 2009) but is much more pronounced, and it is associated with an increase in thylakoid phosphorylation (Armbruster *et al.*, 2013) rather than the decrease that would be expected if PSII core phosphorylation did indeed control the diameter of grana discs as suggested by Fristedt *et al.* (2009). Therefore, the altered interaction between thylakoid membranes seen in *stn8* mutants is unlikely to be due to altered PSII core phosphorylation but rather to altered activity (or oligomerization) of CURT1 proteins, which appear to be reversibly phosphorylatable (Armbruster *et al.*, 2013). But is the total area of appressed thylakoid membrane in a

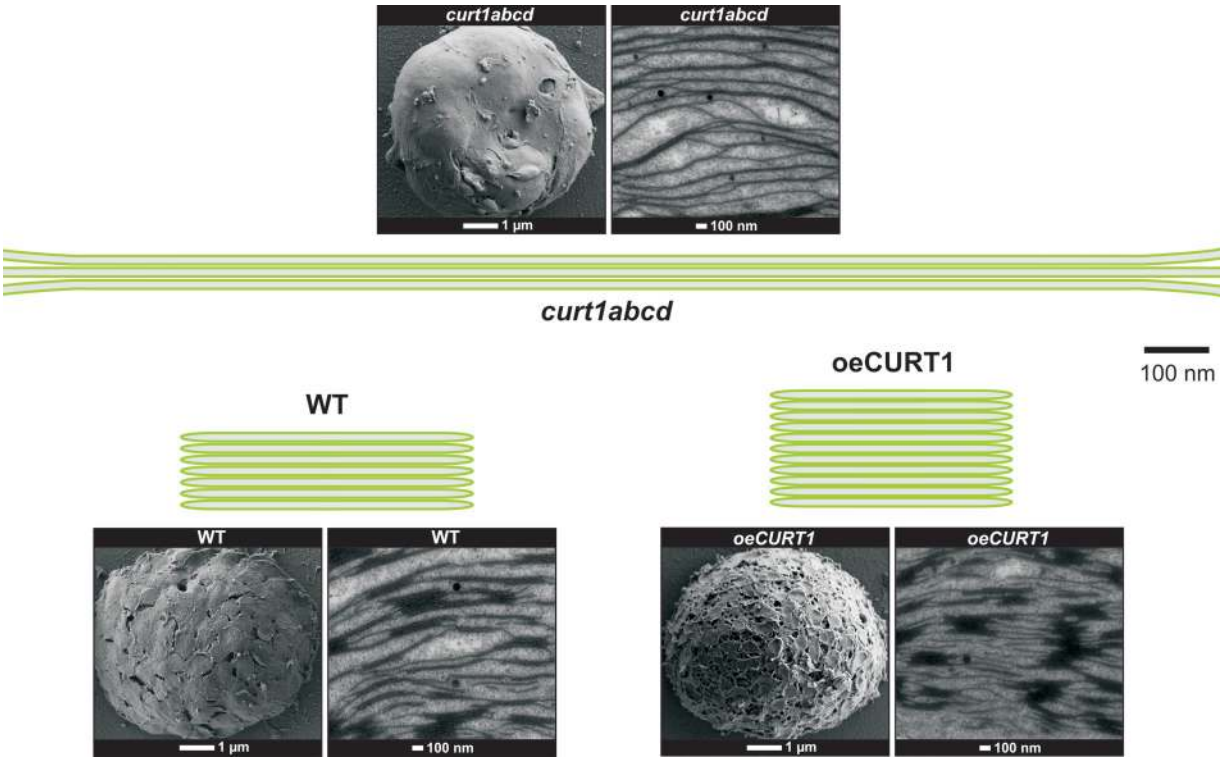

Fig. 3. Effects of CURT1 levels on granum dimensions. Values for granum dimensions of the presented true to scale grana models are derived from Armbruster *et al.* (2013) and are listed in Table 2. Additional scanning and transmission electron micrographs (SEM/TEM) of the chloroplast thylakoid membrane network of *curt1abcd*, wild-type, and oe*CURT1* plants [left, SEM topview, right, TEM cross-section; adapted from Armbruster U, Labs M, Pribil M, Viola S, Xu W, Scharfenberg M, Hertle AP, Rojahn U, Jensen PE, Rappaport F, Joliot P, Dörmann P, Wanner G, Leister D. 2013. Arabidopsis CURVATURE THYLAKOID1 proteins modify thylakoid architecture by inducing membrane curvature. *The Plant Cell* 25, 2661–2678, www.plantcell.org (24 February 2014), Copyright American Society of Plant Biologists] demonstrate the effects of CURT1 levels on grana topology. In *curt1abcd* thylakoids, margins are absent and pseudograna are formed.

pseudogranum in the CURT1-less mutant indeed greater than that in a granum—after all, the increase in disc size is accompanied by a decrease in the number of discs found in a single pseudogranum? The measurements made by Armbruster *et al.* (2013) give an unequivocal answer: the area of thylakoid membrane contained in a pseudogranum is markedly increased in thylakoids of plants without CURT1 proteins compared with wild-type plants (Fig. 3, Table 2). Therefore, in a single pseudogranum of a *curt1abcd* thylakoid, the area of appressed thylakoid membranes is increased, although the total abundance of PSII and LHCII is not altered. Interestingly, the number of grana per chloroplast is altered both in plants without CURT1 and in CURT1A overexpressors (Table 2). Therefore, not only the total extent of margins (estimated on the basis of the total peripheries of discs present in one chloroplast), but also the number of grana stacks, correlates with the amount of CURT1 proteins (Table 2). It is therefore tempting to conclude that these two features are coupled. Moreover, at the level of the chloroplast, not only *curt1abcd* but also CURT1A-overexpressing plants display an increase in thylakoid membrane area located in the (pseudo)grana regions when compared with the wild type. This allows one to conclude that, in addition to proteins in the granum core itself, margin-related factors might exert (indirect) effects on the interaction of thylakoid membranes, thereby shaping the architecture of grana.

Physiological function(s) of grana

The primary purpose of grana as such is unclear, and suggested functions include the prevention of spillover of excitation energy through physical separation of photosystems, fine-tuning of photosynthesis, facilitation of state transitions, switching between linear and cyclic electron flow, and, in particular, enhancement of light harvesting under low light conditions through the formation of large arrays of PSII–LHCII supercomplexes (Trissl and Wilhelm, 1993; Horton, 1999; Mustárdy and Garab, 2003; Dekker and Boekema, 2005; Mullineaux, 2005; Anderson *et al.*, 2008; Daum and Kühlbrandt, 2011; Nevo *et al.*, 2012). However, grana formation also imposes constraints on photosynthesis, such as the requirement for long-range diffusion of electron carriers between PSII and PSI (Mullineaux, 2008; Kirchhoff *et al.*, 2011) and the relocation of PSII between appressed and non-appressed regions during the PSII repair cycle (Mulo *et al.*, 2008). This complex picture of 'advantages' and 'disadvantages' of grana formation led Nevo *et al.* (2012) to conclude that 'it is quite impossible (and, perhaps, unnecessary) to determine which of the aforementioned potential benefits had been the primary driving force behind grana formation. Along the course of evolution, various functions were gradually gained, concomitant with the acquisition of more refined control mechanisms.'

The availability of the *curt1abcd* lines with a primary defect in grana architecture—formation of 'pseudograna' (without margin regions) made up of fewer thylakoid discs with enlarged diameters, as well as of grana consisting of more discs with reduced diameters (in CURT1A overexpressors) (Armbruster *et al.*, 2013) (see Fig. 3)—allows one to address the impact of grana on specific photosynthetic functions. Because thylakoids that lack grana stacks represent the primordial type and are ubiquitous in cyanobacteria, it is not surprising that *curt1abcd* plants are still capable of performing photosynthesis, although they are devoid of canonical grana with margin regions (Armbruster *et al.*, 2013). However, the complex perturbations in the process found in *curt1abcd* plants suggest that the dramatic increase in the area of thylakoid membrane present in the appressed regions of CURT1-less plants (see Fig. 3) does indeed compromise photosynthesis. This in turn

Table 2. *Granum dimensions in lines with different CURT1 levels*

The term (pseudo)granum refers to the appressed regions in *curt1abcd* plants which lack margin regions (see Fig. 3).
Values for the dimensions of grana from *curt1abcd*, CURT1A overexpressors (oeCURT1A), and wild-type plants are from Armbruster *et al.* (2013). The number of discs per (pseudo)granum was calculated according to Kirchhoff *et al.* (2011). The thylakoid membrane area was calculated according to the equation $A=\pi \times d/4$ (with A=circular area and d=diameter). With respect to the total extent of margins, the total periphery of the discs contained in a granum can be taken as the measure. To calculate the total amount of thylakoid membranes present in a single chloroplast, we measured the number of (pseudo)grana stacks in 6–8 chloroplasts for each genotype, and then calculated membrane areas and peripheries per chloroplast.

	curt1abcd	Wild type	CURT1A overexpressor
Granum			
Diameter (μm)	1.656 ± 0.075	0.448 ± 0.016	0.374 ± 0.006
Height (μm)	0.056 ± 0.002	0.113 ± 0.005	0.173 ± 0.006
No. of layers in (pseudo)grana	3.4	6.9	10.6
Membrane area including end membranes (μm^2)	14.8	2.2	2.3
Membrane area without end membranes (μm^2)	10.5	1.9	2.1
Total disc peripheries (μm)	–	9.8	12.5
Chloroplast			
No. of (pseudo)grana	13.3 ± 3.3	34.6 ± 8.9	61.1 ± 14.8
Membrane area including end membranes (μm^2)	196.8	75.6	142.5
Membrane area without end membranes (μm^2)	139.5	64.7	129.1
Total disc peripheries (μm)	–	337.6	761.9

is compatible with the idea that important features of photosynthesis in land plants are indeed dependent on, or at least facilitated by, the lateral heterogeneity provided by grana. One possibility is that, in CURT1-less mutants, the dearth of intermediate regions between appressed regions and stroma lamellae at which processes such as state transitions, PSII repair, and cyclic electron flow normally take place could account for the subtle alterations observed by Armbruster *et al.* (2013). Moreover, because of the increased diameter of pseudograna in the *curt1abcd* mutants, long-range diffusion of electron carriers between PSII and PSI might become limiting, and this could explain the observed drop in linear electron flow.

The plasticity of thylakoid ultrastructure

Variations in the spectral composition and intensity of light, and the duration of light exposure, can affect the integrity and efficiency of the photosynthetic complexes in thylakoids. Thus land plants have evolved a variety of adaptations that optimize the excitation of their photosystems and protect them from light-induced damage. These range from developmental adjustments of plant growth [as in the case of shade avoidance reactions (Franklin, 2008)] and positioning of leaves at an appropriate angle with respect to incident solar radiation (Inoue *et al.*, 2008), to intracellular responses leading to the redistribution of chloroplasts within the cytosol. This last process is triggered by blue light and causes chloroplasts to accumulate in a plane either perpendicular or parallel to the direction of incident light, depending on whether light harvesting needs to be increased or decreased (Wada *et al.*, 2003). At the level of chloroplast ultrastructure, the thylakoid membrane turns out to be a highly flexible system, which can respond quickly to changes in ambient light conditions.

As described above, electron microscopy-based studies have led to a variety of 3-D models for grana (see Fig. 1). However, these models represent snapshots and do not capture the dynamics of the thylakoid system. Exposure of this apparently static membrane structure to different light conditions reveals that it is in fact highly malleable (Fig. 4). Thus, to maximize use of low light levels, the numbers of layers in grana stacks are increased (Anderson, 1986), while high light intensities lead to significant reduction in the diameter, and to partial transversal unstacking, of grana discs (Fristedt *et al.*, 2009; Khatoon *et al.*, 2009; Herbstová *et al.*, 2012). The reduction in grana diameter and modification of the stacked structure under photo-damaging light conditions is thought to facilitate PSII repair in several ways. Damaged PSII complexes, previously embedded within the grana stack, become more accessible to auxiliary proteins, such as FtsH (Nixon *et al.*, 2005), involved in D1 degradation, which are otherwise excluded from the grana stacks due to steric hindrance (Kirchhoff, 2013*b*). Furthermore, lateral shrinkage reduces the time required for damaged D1 to diffuse from the grana core to the margins. In addition, an increase in protein mobility upon exposure to bright light enhances diffusion and turnover of D1 (Herbstová *et al.*, 2012; Kirchhoff, 2014) (Fig. 5A). The partial destacking of grana should further ease migration of LHCII from grana-located PSII to PSI in unstacked thylakoid membranes, a process that is essential for state transitions. Furthermore, swelling of the thylakoid lumen is observed upon dark to light transition, which should facilitate the diffusion of lumenal photosynthetic electron carriers such as plastocyanin (Kirchhoff *et al.*, 2011) or of components involved in the degradation of damaged PSII components (such as Deg proteases) (Kirchhoff, 2013*b*) (Figs 4, 5A). This osmotic expansion in the light is thought to result from an influx of Cl^- and Ca^{2+} ions (Ettinger *et al.*, 1999; Spetea and Schoefs, 2010). While some of these structural rearrangements seem to promote molecular photo-protective mechanisms such as the turnover of photodamaged D1 (Herbstová *et al.*, 2012; Kirchhoff, 2013*b*), other structural changes seem to occur as a consequence of the redistribution of protein components within the thylakoid membrane driven by adaptation mechanisms, including state transitions and other processes, involving reversible protein phosphorylation.

In the following, we turn to the regulatory mechanisms, acting at the molecular and supramolecular levels and operating predominantly in a reversible protein

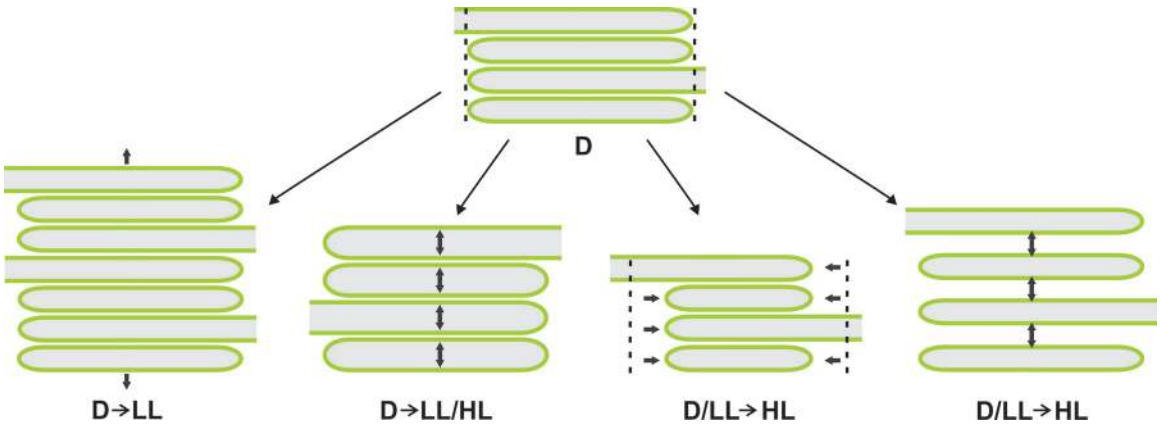

Fig. 4. Schematic depiction of the effects of changes in light conditions on thylakoid membrane organization (adapted from Kirchhoff H. 2013*a*. Architectural switches in plant thylakoid membranes. *Photosynthesis Research* 116, 481–487, with kind permission from Springer Science and Business Media). While the switch from dark to light generally induces osmotic swelling of the thylakoid lumen (D→LL/HL), low light specifically promotes an increase in the number of layers per granum (D→L). Structural changes upon exposure to high light levels involve a decrease in grana diameter as well as vertical unstacking of adjacent membrane layers within the granum (D/LL→HL). D, dark; LL, low light; HL, high light.

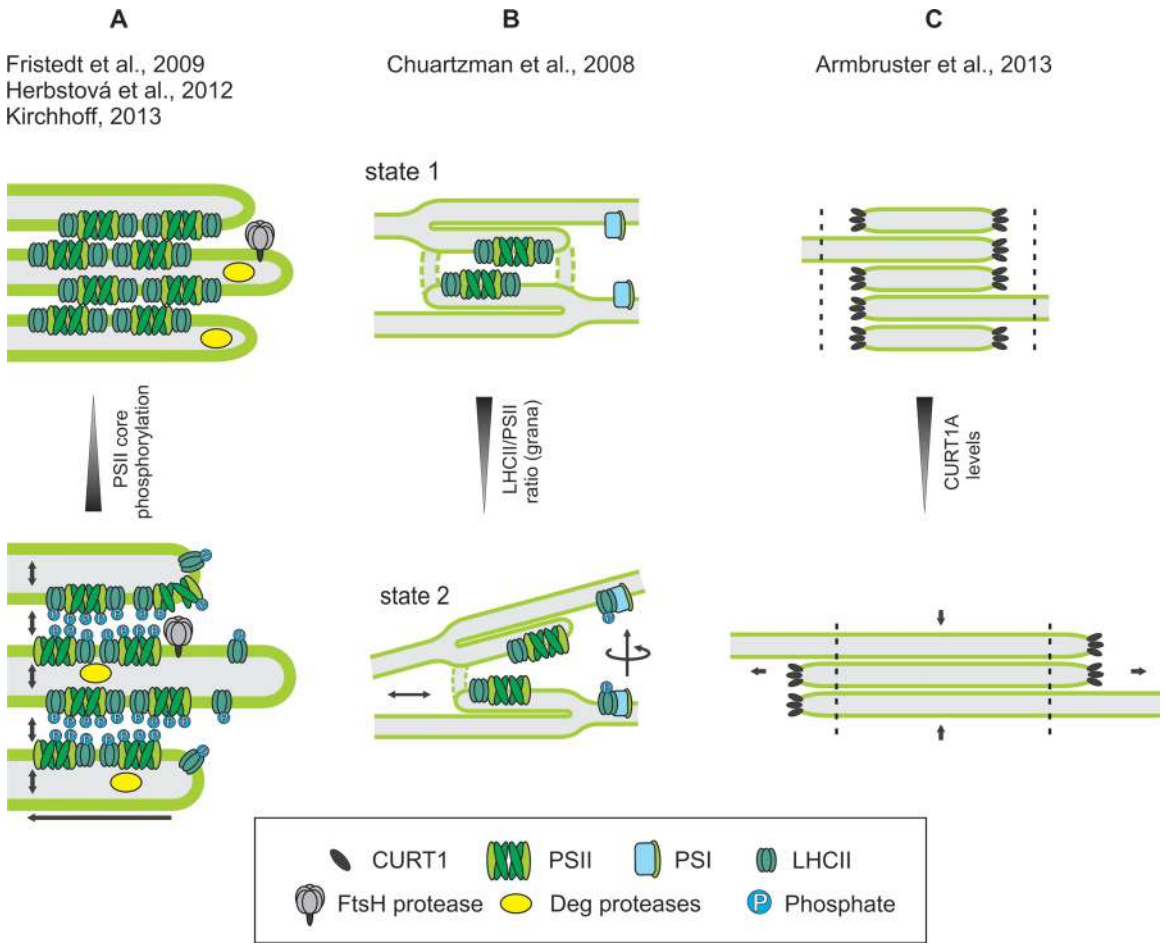

Fig. 5. Processes driven by molecular switches activated under different light conditions. Regulatory mechanisms include events based on protein modifications and/or redistribution of protein components within the thylakoid membrane, which initiate macroscopic rearrangements. (A) Cartoon showing the dynamic interplay between phosphorylation-dependent molecular processes and macroscopic rearrangements at the thylakoid margins under exposure to intense light. Light exposure initiates osmotic swelling of the thylakoids, which facilitates diffusion of lumenal proteins (e.g. electron carriers) and enables lumen-located proteases to penetrate into the grana structure to facilitate D1 turnover. Phosphorylation of PSII core proteins induced by high light levels is thought to trigger vertical unstacking of adjacent membrane layers through electrostatic repulsion, thus promoting lateral migration of photodamaged PSII to unstacked stroma lamellae. Concomitantly, the FtsH protease, which protrudes into the stroma, obtains access to the grana regions and lateral shrinkage of the grana diameter is induced, which also promotes photoprotective mechanisms. (After Fristedt R, Willig A, Granath P, Crèvecoeur M, Rochaix JD, Vener AV. 2009. Phosphorylation of photosystem II controls functional macroscopic folding of photosynthetic membranes in Arabidopsis. *The Plant Cell* 21, 3950–3964. www.plantcell.org (last accessed 24 February 2014), Copyright American Society of Plant Biologists.) (B) Remodelling of the granum–stroma assembly during the state 1–2 transition in the context of the fork model of Shimoni *et al.* (2005). Bifurcated stroma lamellar sheets are interconnected by membrane bridges. Upon migration of LHCII from grana to stroma, grana margins become destabilized, and adjacent layers retract from each other, causing some of the membrane bridges to break. This initiates a shape transition that potentially involves rotation and vertical and lateral membrane motions. (After Chuartzman SG, Nevo R, Shimoni E, Charuvi D, Kiss V, Ohad I, Brumfeld V, Reich Z. 2008. Thylakoid membrane remodeling during state transitions in Arabidopsis. *The Plant Cell* 20, 1029–1039. www.plantcell.org (last accessed 24 February 2014), Copyright American Society of Plant Biologists.) (C) Schematic model of the effects of variations in amounts of CURT1 proteins on membrane stacking. CURT1 amounts are positively correlated with the degree of membrane curvature and number of grana layers. In this respect, potential phosphorylation-induced changes in CURT1 structure or oligomerization state might represent an additional layer of regulation.

phosphorylation-dependent manner, which account for the plasticity of the thylakoid ultrastructure.

Alterations in protein complex composition and supramolecular organization

The protein composition of thylakoids (see Fig. 2) can be modulated by alterations in the relative levels of resident complexes, or the stoichiometry of their subunits (Anderson, 1986). For instance, exposure to low light leads to an increase in the ratio of LHCII to PSII and Cyt b_6f (Anderson, 1986; Staehelin, 1986; Kirchhoff *et al.*, 2007). Such changes in protein stoichiometry represent long-term acclimation processes, which require tight coordination of gene expression between the chloroplast and the nucleus. However, the nature of the retrograde signalling pathways that control such responses is still hotly debated (Kleine *et al.*, 2009; Pfannschmidt, 2010). Besides these alterations in protein stoichiometry, differential localization of protein complexes within the thylakoid membrane also exerts a direct influence on its ultrastructure. In particular, the assembly of PSII and LHCII into super- and megacomplexes (Dekker and Boekema, 2005; Kouřil *et al.*,

2012) significantly contributes to the formation of grana stacks. Under unfavourable conditions such as low temperature or low light (Garber and Steponkus, 1976; Kirchhoff *et al.*, 2007), these higher order PSII–LHCII complexes can form semi-crystalline arrays that may occasionally span the entire grana disc (Staehelin, 1986; Dekker and Boekema, 2005; Goral *et al.*, 2012). Such PSII macro-structures seem to be susceptible to even minor structural perturbations, as in the case of a lack of CP29 or increased levels of PsbS (Goral *et al.*, 2012). Thus, while the presence of CP29 seems to be essential for formation of PSII–LHCII supercomplexes (Kouřil *et al.*, 2012), PsbS appears to suppress the assembly of large PSII arrays (Goral *et al.*, 2012). The latter observation is in line with the idea that non-photochemical quenching (NPQ) involves partial disassembly of PSII–LHCII supercomplexes to allow LHCII aggregation (Betterle *et al.*, 2009; Johnson *et al.*, 2011).

PSII protein phosphorylation and D1 turnover

Thylakoid phosphorylation is mainly mediated by the two protein kinases STN7 and STN8 (Bellafiore *et al.*, 2005; Bonardi *et al.*, 2005; Vainonen *et al.*, 2005). While STN7 primarily catalyses LHCII phosphorylation (see the following section), STN8 is predominantly responsible for the phosphorylation of PSII core proteins (CP43, D1, D2, and PsbH), especially under high light conditions (Bonardi *et al.*, 2005; Vainonen *et al.*, 2005; Tikkanen *et al.*, 2010). Recent studies on *stn7*, *stn8* and *stn7 stn8* double mutants have revealed that phosphorylation of PSII core proteins plays a role in the PSII repair cycle during photoinhibition by facilitating migration of damaged PSII reaction-centre proteins from grana to stroma lamellae, where they undergo degradation (Tikkanen *et al.*, 2008). The PSII repair cycle involves a sequence of steps that includes (de-)phosphorylation of PSII subunits, disassembly and reconstruction of super- and holocomplexes, and degradation/*de novo* synthesis of D1 (Aro *et al.*, 1993; Kato and Sakamoto, 2009; Pesaresi *et al.*, 2011). Note, however, that protein phosphorylation by STN7 and STN8 is not essential for the PSII repair cycle (Bonardi *et al.*, 2005), but alters its dynamics, such that disassembly of PSII supercomplexes is impaired in the absence of PSII phosphorylation (Tikkanen *et al.*, 2008). Therefore, it was suggested that STN8-dependent phosphorylation of PSII core proteins modulates thylakoid ultrastructure by enhancing the lateral mobility of thylakoid membrane protein complexes and thus promoting PSII repair (Fristedt *et al.*, 2009; Goral *et al.*, 2010) (Fig. 5A). Indeed, no such increase in mobility is observed in isolated grana membranes, or in *stn8* and *stn7 stn8* mutants lacking thylakoid phosphorylation (Goral *et al.*, 2010). Fristedt *et al.* (2009) proposed that the observed increase in the diameter and density of grana stacks in the *stn8* and *stn7 stn8* mutants reduces lateral diffusion of proteins, including that of photodamaged D1 and of the bulky FtsH complex, which is essential for its degradation (Nixon *et al.*, 2005) and was reported to be spatially separated from PSII in STN8-deficient mutants due to steric hindrance (Fig. 5A). In agreement with this, *A. thaliana* plants overexpressing STN8 were less sensitive to intense light and exhibited alterations in thylakoid ultrastructure, with grana stacks containing more layers and reduced amounts of PSII supercomplexes (Wunder *et al.*, 2013). An additional architectural adaptation that occurs upon exposure to high light levels is a decrease (of ~20%) in grana diameter. Because no such structural alterations were observed in the *stn8* and *stn7 stn8* mutants (Herbstová *et al.*, 2012), this decrease can be attributed to the effect of PSII core phosphorylation (Fig. 5A).

LHCII phosphorylation and state transitions

State transitions in plants serve to adjust the absorption properties of PSII and PSI at low light intensities so as to optimize utilization of the light available (Allen and Forsberg, 2001). The state 1–2 transition involves the phosphorylation-dependent detachment of LHCII from PSII (in the grana) and its diffusion to PSI (in the stroma lamellae). This reaction is primarily catalysed by the LHCII kinase STN7 (Bellafiore *et al.*, 2005; Bonardi *et al.*, 2005), although there is some overlap in substrate specificity between STN7 and the PSII core kinase STN8 (Bonardi *et al.*, 2005). Dephosphorylation of LHCII, which is required to allow LHCII to move back to PSII and thereby initiates the reverse (2–1) transition, is catalysed by the protein phosphatase TAP38/PPH1 (Pribil *et al.*, 2010; Shapiguzov *et al.*, 2010). Moreover, disassembly of the PSII–LHCII supercomplex appears to promote state transitions. Thus, destabilization of PSII supercomplexes, either genetically in the *psb27* mutant or by light treatment, accelerates state transitions (Dietzel *et al.*, 2011).

Because LHCII is thought to be the major stabilizer of appressed grana domains (Dekker and Boekema, 2005), the movement of LHCII between grana (PSII) and stroma (PSI) should alter stacking of the grana of higher plants. However, the extent of structural rearrangement that occurs within the thylakoid membrane upon state transitions is disputed. Treatment of thylakoids with dilute salt solutions, which is thought to mimic the state 1–2 transition, leads to extensive structural rearrangements including complete destacking of the appressed membrane regions (Izawa and Good, 1966; Murakami and Packer, 1971). Based on these observations, the movement of LHCII from grana to stroma lamellae was proposed to bring about similar effects (Ryrie, 1983; Arvidsson and Sundby, 1999). However, *in vivo* studies based on light-induced state transitions revealed only moderate differences in membrane stacking that remained restricted to grana margins and typically did not exceed 10–20% (Kyle *et al.*, 1983; Drepper *et al.*, 1993; Delosme *et al.*, 1996; Rozak *et al.*, 2002; Mustárdy and Garab, 2003; Shimoni *et al.*, 2005). Therefore, assuming that LHCII phosphorylation occurs at the grana margins, it was proposed that LHCII migration between grana and stroma lamellae is associated with changes in the local lipid–protein composition, which in turn cause retraction of the membrane layers and destabilize the grana–stroma interface (Chuartzman *et al.*, 2008). Eventually a limited fraction of membrane bridges break and undergo structural rearrangements, such as retraction of neighbouring layers along the granum axis or rotational movements, that cause the layers to be displaced from the granum core

(Chuartzman *et al.*, 2008) (Fig. 5B). This then leads to destacking and fragmentation of grana.

Effects of reversible phosphorylation on CURT1 proteins

Thylakoid protein phosphorylation has previously been associated with changes in grana architecture following the observation that plants with reduced thylakoid phosphorylation (*stn7 stn8*) form grana with fewer but broader membrane discs (Fristedt *et al.*, 2009). Conversely, grana stacks of *tap38* mutant plants that show increased thylakoid phosphorylation assemble more membrane layers than the wild type (Armbruster *et al.*, 2013). Interestingly, the effect of a decrease in thylakoid phosphorylation on grana architecture strongly resembles that seen when levels of CURT1 are reduced (Fig. 5A, C). However, instead of showing reduced levels of thylakoid phosphorylation, the CURT1 mutants display increased LHCII and PSII phosphorylation (Armbruster *et al.*, 2013). This suggests that CURT1-dependent alterations in grana ultrastructure either do not involve PSII core phosphorylation or over-ride such effects. In fact, there is good evidence that CURT1-dependent thylakoid plasticity is actually regulated via reversible phosphorylation. Based on phospho-proteomics studies, the major CURT1 proteins can indeed be reversibly phosphorylated (Armbruster *et al.*, 2013). More specifically, CURT1A is potentially phosphorylated at three different positions (Ser64, Ser65, and Thr68) and CURT1B at two residues (Thr65 and Thr66) (Durek *et al.*, 2010). It is possible that in *stn7 stn8* double mutants CURT1 proteins are dephosphorylated, with concomitant effects on their structure and/or oligomerization behaviour, which in turn alter thylakoid ultrastructure (Armbruster *et al.*, 2013).

Biogenesis of thylakoids

The sophisticated thylakoid ultrastructure seen in mature chloroplasts is not observed in the proplastids found in dark-grown tissues, but its formation is rapidly initiated upon exposure to light. The process of thylakoid biogenesis requires the coordinated assembly of lipids, proteins, and chlorophylls, which together account for >98% of the mass of the thylakoid membrane (Murphy, 1986). Strikingly, lipids make up only ~30% of the membrane surface (Kirchhoff *et al.*, 2002). Of these, >50% cannot normally form bilayers under the conditions prevailing in thylakoids (Webb and Green, 1991). Studies on mutant plants have been most helpful in elucidating the interdependence of component assembly during thylakoid biogenesis.

De-etiolation

The membrane system in proplastids is much simpler than that found in mature chloroplasts. Its simple vesicular structures contain only small amounts of proteins linked to photosynthesis (Adam *et al.*, 2011). In the absence of light, the proplastid matures into an etioplast, whose inner membrane forms a semi-crystalline network of interconnected tubules called a prolamellar body (Rosinski and Rosen, 1972). The prothylakoids develop as extensions of the prolamellar body, but contain lower amounts of monogalactosyldiacylglycerol (MGDG), thus allowing them to form a planar bilayer structure (Selstam and Sandelius, 1984) (Fig. 6A). The prolamellar body is photosynthetically inactive, but analysis of its proteome has identified 64 proteins that are linked to the photosynthetic light reactions, the Calvin cycle, protein synthesis (including chaperones), and pigment biosynthesis (Blomqvist *et al.*, 2008). By this point, the ATP synthase is fully assembled (Plöscher *et al.*, 2011) and the dimeric Cyt b_6f complex only lacks mature chlorophyll *a* (Reisinger *et al.*, 2008). In contrast, PSII biogenesis is arrested at a pre-complex stage, and PSI assembly most probably occurs later in the greening process (Müller and Eichacker, 1999). In etioplasts, LHCs are not yet inserted into the membrane (Kuttkat *et al.*, 1997). The most abundant protein in prolamellar bodies is the NADPH:protochlorophyllide oxidoreductase (POR), and mutants that lack this enzyme accumulate precursors of protochlorophyllide and possess no prolamellar bodies (Lebedev *et al.*, 1995; Sperling *et al.*, 1998). Further major constituents of the prolamellar body are protochlorophyllide, NADPH, and the non-bilayer-forming lipid MGDG (Selstam and Sandelius, 1984; Adam *et al.*, 2011). The high content of POR (90% of the protein content) is compatible with the cubic lipid structure adopted by MGDG and thus accounts for the semi-crystalline form of prolamellar bodies (Selstam and Sandelius, 1984; Ryberg and Sundqvist, 1988). As the initial step in light adaptation, protochlorophyllide is reduced by POR to chlorophyllide and later esterified into chlorophyll (Von Wettstein *et al.*, 1995). At the same time, the prolamellar body loses its semi-crystalline structure and the extruded lamellae align in parallel throughout the stroma (Fig. 6B, C). Whether the lipids of the prolamellar body are directly incorporated into the prothylakoids or are transferred via vesicles is unclear (Rosinski and Rosen, 1972; Adam *et al.*, 2011). The transformation of the semi-crystalline prolamellar body into planar thylakoids (Fig. 6D) can take from 1 h to over a day.

Lipid incorporation

The thylakoid membrane contains five major lipids. The non-bilayer-forming MGDG accounts for 52% of total lipids by weight (Kirchhoff *et al.*, 2002). Due to its small headgroup, MGDG forms inverted hexagonal (H_{II}) structures in solution at physical pH and temperature (Goss and Wilhelm, 2010). Digalactosyldiacylglycerol (DGDG) with 27%, sulphoquinovosyldiacylglycerol (SQDG) with 15%, phosphatidylglycerol (PG) with 3%, and phosphatidylcholine (PC) with 3% account for the rest (Webb and Green, 1991; Kirchhoff *et al.*, 2002). Because the synthesis of all of these lipids is finalized in the chloroplast envelope, a mechanism for their continuous transport to thylakoids must exist (Jouhet *et al.*, 2007; Benning, 2008). Whether this transfer of lipids occurs via (i) a vesicular pathway; (ii) soluble glycerolipid transfer proteins; or (iii) invaginations that directly connect the envelope to thylakoids is not clear (Fig. 6D) (Holthuis and Levine, 2005; Jouhet *et al.*, 2007). A mutant with a defective

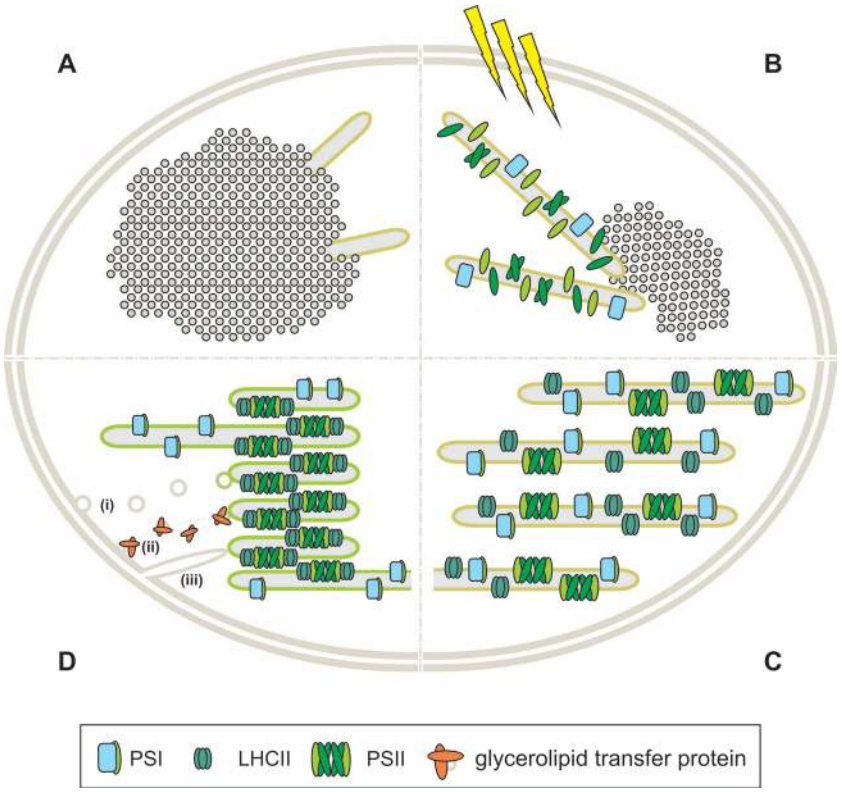

Fig. 6. Schematic overview of the light-dependent de-etiolation process and the biogenesis of thylakoid membranes. This simplified scheme focuses on PSII, PSI, and LHC. (A) The etioplast contains prothylakoids and the semi-crystalline prolamellar body. The latter is mainly composed of MGDG, POR, and protochlorophyllide. MGDG and POR together form the cubic lipid structure that makes up the prolamellar body. (B) Etio-chloroplast stage. The de-etiolation process is initiated by exposure to light and the light- and NADPH-dependent reduction of protochlorophyllide by POR. The semi-crystalline prolamellar body disassembles. Whether the lipids of the prolamellar body are incorporated into the maturing prothylakoids directly or via vesicular or tubular intermediates is unknown. Mainly monomeric proteins are incorporated into the developing thylakoids. (C) The lamellar structures align in parallel within the chloroplast. With the formation of protein complexes, the thylakoids enter a photoactive state. (D) The grana stacks characteristic of mature thylakoids form upon incorporation of mega- and supercomplexes. It is assumed that lipids reach thylakoids only via (i) a vesicular pathway, but might also be supplied by (ii) soluble glycerolipid transfer proteins or (iii) via invaginations of, or direct contact sites with, the inner envelope. This schematic representation of the processes involved in the de-etiolation of etioplasts does not do justice to the complexity of the intermediate steps (reviewed in Solymosi and Schoefs, 2010). For example, grana stacks and prolamellar bodies have been observed in the same chloroplast in electron micrographs (Solymosi and Schoefs, 2010).

MGDG synthase 1 (*mgd1*) is unable to produce photosynthetically active membranes, but shows invaginations of the inner envelope (Kobayashi *et al.*, 2013). However, the emergence in cold-incubated plants of vesicles (Morré *et al.*, 1991) that resemble the COPII vesicles seen in the cytosol under cold conditions when the energy requirement for fusion with target membranes increases (Saraste *et al.*, 1986; Morré *et al.*, 1989) points to a role for vesicular traffic in the biogenesis and maintenance of thylakoids (Vothknecht and Westhoff, 2001).

Mutants without the 'vesicle-inducing protein in plastids' (VIPP1) lack the aforementioned cold-induced vesicles and are defective in thylakoid biogenesis (Kroll *et al.*, 2001). Because it is found both at the inner envelope and at the thylakoids (Li *et al.*, 1994), a role for VIPP1 in the formation of vesicles that transport lipids and hydrophobic carotenoids to the thylakoids has been proposed (Kroll *et al.*, 2001). Mutants for the cyanobacterial homologue of VIPP1, PspA (bacterial phage shock protein A), also show a thylakoid-defective phenotype (Westphal *et al.*, 2001), and a maintenance function for membrane integrity was suggested for it (Hankamer *et al.*, 2004; Standar *et al.*, 2008). Note that the central α-helical domain, conserved between PspA and Vipp1, is responsible for formation of an oligomeric ring structure (Aseeva *et al.*, 2004), whereas the N-terminal α-helix mediates lipid binding and assembly of a high molecular weight complex (Otters *et al.*, 2013). The dynamics of this complex are controlled by the HSP70B–CDJ2–CGE1 chaperones (Liu *et al.*, 2005; Liu *et al.*, 2007), but HSP90 may promote the disassembly of the multimer and, in its absence, only a few thylakoid membranes are formed (Feng *et al.*, 2014). Other findings hint that VIPP1 might function like its bacterial homologue, namely acting to maintain the chloroplast envelope instead of inducing vesicles, thus having a protective rather than a driving effect on thylakoid biogenesis (Zhang *et al.*, 2012). It has been speculated that thylakoid-associated VIPP1 has a similar function, but conclusive proof is still missing (Vothknecht *et al.*, 2012; Zhang and Sakamoto, 2013). Moreover, VIPP1 was also found to enhance binding of substrates for the cpTat import pathway (Lo and Theg, 2012). Intriguingly, recent results point to a role for VIPP1 in the assembly of thylakoid core complexes (Nordhues *et al.*, 2012). Based on these findings, Rütgers and Schroda (2013) have presented a model in which

VIPP1 fulfils a structural role within thylakoid centres, which are considered as sites from which thylakoid membranes emerge and at which the biogenesis of PSII at least is thought to occur. Furthermore, VIPP1 could create microdomains in the membrane that facilitate the accumulation of specific lipids that, in turn, aid in the function of translocases (Lo and Theg, 2012; Rütgers and Schroda, 2013).

VIPP1 apparently does not play a role in vesicular transport in mature chloroplasts, but several other proteins remain as candidates for such factors. Thus, a bioinformatics approach has identified chloroplast-located homologues of the COPII vesicular pathway between the endoplasmic reticulum and Golgi apparatus (Andersson and Sandelius, 2004). One essential component for assembly of the COPII coat is the GTPase Sar1, whose chloroplast-located homologue cpSar1 also shows GTPase activity *in vitro* and has been linked to thylakoid biogenesis (Garcia *et al.*, 2010). Although a direct connection with vesicle coat assembly could not be demonstrated, the protein's presence at the inner envelope and in the stroma is compatible with a function in vesicle initiation. Furthermore, cpSar1 has been detected around cold-induced vesicles (Garcia *et al.*, 2010). While *cpSar1* knock-out mutants show developmental arrest before greening, *cpSar1* RNAi (RNA interference) lines show an interesting intermediate phenotype with respect to thylakoid biogenesis. In these lines, plastids contain vesicles of various sizes that eventually coalesce and form the typical mature grana stacks (Garcia *et al.*, 2010).

The dynamin family member FZL is also localized at the envelope and thylakoids, and shows GTPase activity, but in *FZL* knock-out plants disruption of thylakoid ultrastructure is less severe (Gao *et al.*, 2006) than in *cpSar1* knock-outs. Although grana stacks are disorganized and vesicles accumulate, FZL is believed to play a more prominent role later in thylakoid development (Gao *et al.*, 2006; Adam *et al.*, 2011).

The THF1 (THylakoid Formation1) protein is also assumed to be involved in vesicular trafficking because in *thf1* mutants white/yellow patches appear that completely lack grana stacks or any form of thylakoid membrane but accumulate membrane vesicles (Wang *et al.*, 2004). THF1 is identical to Psb29, which is involved in PSII biogenesis (Keren *et al.*, 2005). This finding is corroborated by the observation that *thf1* mutants retain a PSII–LHCII supercomplex in the dark, which implies an important role for THF1/Psb29 in PSII dynamics (Huang *et al.*, 2013). Therefore, it cannot be excluded that THF1/Psb29 might be involved in the fusion of PSII-loaded vesicles emerging from the envelope (Khan *et al.*, 2013), although this seems at variance with the continuous influence of THF1 on leaf development including leave senescence (Huang *et al.*, 2013).

Given the complexity of COPII vesicular transport, a mechanism dedicated solely to the transport of lipids from the envelope to thylakoids is hard to imagine, especially since the non-bilayer-forming nature of MGDG would complicate such mechanisms. In this context, two observations are of interest: (i) the MDGD:DGDG ratio is three times lower in developing than in mature thylakoids (Andersson *et al.*, 2001) and (ii) the existence of non-bilayer structures in thylakoids and their ability to exchange lipids with the bilayer phase (Krumova *et al.*, 2008). Thus, the integration of high concentrations of MGDG into a lipid bilayer relies on the presence of membrane proteins. If the protein to MGDG ratio is lowered, MGDG cannot be kept within the bilayer, but migrates into non-bilayer structures. However, whether bilayer and non-bilayer phases can co-exist in vesicles too remains speculative. Alternatively, it was suggested that a vesicle pathway might also transport non-lipid components (Westphal *et al.*, 2003; Benning, 2009). This idea is in line with the identification of the plasma membrane as the location of initial photosystem biogenesis in cyanobacteria (Zak *et al.*, 2001), although this apparently does not hold for *A. thaliana* (Che *et al.*, 2013). In an ongoing bioinformatics analysis, the search for components of the vesicular transport mechanism in plastids has been expanded to associated factors (Khan *et al.*, 2013). In this study, chloroplast-targeted homologues of coat proteins, cargo receptors, tethering factors, and SNAREs were identified. Some 80% of the putative cargo proteins could be linked to functions in thylakoids such as biogenesis, stress responses, and photosynthesis (Khan *et al.*, 2013).

Despite the lack of conclusive experimental proof, the evidence for a vesicular transport system within the chloroplast cannot be easily dismissed (Brandizzi, 2011).

The role of protein complexes in thylakoid biogenesis

The vast majority of the thylakoid surface is occupied by protein complexes, which account for >70% of the total thylakoid membrane area (Kirchhoff *et al.*, 2002). Thus, it seems likely that thylakoid biogenesis is influenced by the insertion of protein complexes into the lipid bilayer matrix. Most of the thylakoid proteins are encoded in the nucleus and synthesized in the cytosol, and must be post-translationally imported into the chloroplast. The pathways mediating this transport, and its evolution and regulation, have been extensively reviewed (Gutensohn *et al.*, 2006; Strittmatter *et al.*, 2010; Shi and Theg, 2013). Recently, it was suggested that lumenal proteins are also essential for thylakoid biogenesis (Shipman-Roston *et al.*, 2010; Järvi *et al.*, 2013). Their proper maturation may be a key step in the assembly of thylakoids, as plants mutant for the processing peptidase PLSP1, which is involved in the maturation of lumenal proteins (such as OE33, OE23, and plastocyanin), have been shown to accumulate large amounts of vesicles in the stroma but fail to develop intact thylakoids in adult plants (Inoue *et al.*, 2005; Shipman and Inoue, 2009; Shipman-Roston *et al.*, 2010). Here, the critical step seems to be the removal of the thylakoid-transfer signal. Without its removal, certain newly imported proteins are not released from the thylakoid membrane (Frielingsdorf and Klösgen, 2007) and are subsequently degraded (Midorikawa and Inoue, 2013).

Generally, it is difficult to determine unambiguously the importance of integral membrane proteins for thylakoid biogenesis, since their absence results in significant perturbation of photosynthetic activity. In the following, defects in the assembly of the major thylakoid protein complexes will be reviewed in the context of their effects on thylakoid biogenesis.

PSI

Mutants without PSI are incapable of photoautotrophic growth. First identified in a series of high chlorophyll fluorescence (hcf) mutants (Meurer *et al.*, 1996), *hcf101* was depleted of PSI and showed an impaired thylakoid ultrastructure completely devoid of stroma lamellae (Stöckel and Oelmüller, 2004). HCF101 was found to be involved in the provision of Fe–S clusters required for PSI assembly (Lezhneva *et al.*, 2004; Schwenkert *et al.*, 2010). Interestingly, other mutants specifically lacking PSI form fragmentary stroma lamellae but still express near wild-type levels of the light-harvesting complexes. These include strains defective for the PSI assembly factor PPD1 (PsbP-domain protein1) (Liu *et al.*, 2012), PSI-F, a subunit of PSI (Haldrup *et al.*, 2000), and Pale yellow green7 (Pyg7) (Stöckel *et al.*, 2006), as well as *hcf101*, *hcf113*, and *hcf140* (Amann *et al.*, 2004). Therefore, it can be concluded that the presence of PSI is essential for thylakoid biogenesis, more specifically the formation of the stroma lamellae.

PSII

The aforementioned PSII assembly mutant *hcf136* forms enlarged and denser grana stacks, while light-harvesting complexes assemble normally (Meurer *et al.*, 1998). In Low PSII Accumulation1 (LPA1) lines, which retain 20% of the wild-type PSII amount, grana stacks are shorter and thinner, but the overall effect on thylakoid ultrastructure is less severe (Peng *et al.*, 2006). In the absence of *AtCtpA*, a protein required for maturation of the PSII reaction centre protein D1, no functional PSII complexes, and few grana stacks, could be assembled (Che *et al.*, 2013). Conversely, overexpression of maize plastidial transglutaminase in tobacco increased the numbers of PSII centres in the appressed grana, leading to larger grana stacks and reduced stroma lamellae (Ioannidis *et al.*, 2009). The lack of ATAB2 (Arabidopsis homologue of Chlamydomonas Tab2), which is presumably involved in the biogenesis of both photosystems, results in an intermediate thylakoid phenotype (Dauvillée *et al.*, 2003; Barneche *et al.*, 2006). In *atab2* mutants, PSI complexes are absent, PSII is decreased 5-fold, while the Cyt b_6f and ATPase complexes are expressed normally, leading to a significant decrease in stroma lamellae and a general decrease in thylakoid membrane content (Barneche *et al.*, 2006).

Light-harvesting complex proteins (LHCPs)

Post-translational insertion of the LHCPs into thylakoids is mediated by the signal recognition particle (cpSRP) pathway (Schünemann, 2004). In the case of LHCPs, this works in close cooperation with FtsY and Albino3 (ALB3) (Tu *et al.*, 1999; Moore *et al.*, 2000; Woolhead *et al.*, 2001), with ALB3 being responsible for cpSRP-dependent LHCP integration into the thylakoid membrane (Bals *et al.*, 2010; Falk *et al.*, 2010). The importance of ALB3, and hence of the LHCPs, for the biogenesis of thylakoid membranes is striking. In the *alb3* mutant, a significant loss in thylakoid membrane and grana stacking is observed (Sundberg *et al.*, 1997). As mentioned above, the impact on grana formation of a specific lack of LHCII trimers or alterations in their subunit composition is less severe.

The assembly of the LHCIIs also relies on the incorporation of chlorophyll *b* (Horn *et al.*, 2007). In a mutant devoid of chlorophyll *b* (*ch1-3*), the concentration of LHCII was decreased, with no LHCII trimers detectable (Kim *et al.*, 2009). This led to smaller chloroplasts and a 30% decrease in numbers of grana per chloroplast area. A cross of *ch1-3* and *lhcb5* showed a further decrease in LHCII monomers and resulted in a loss of >60% grana area (Kim *et al.*, 2009). This decrease in grana stacking due to reduced LHCII levels was attributed to a decline in van der Waals attraction, lower electrostatic interaction between opposite charges across the partitioning gap, and impaired formation of PSII–LHCII aggregates, which together appear to exert stronger negative effects on grana formation than the positive effects caused by the weaker electrostatic repulsion due to the lack of LHCII (Chow *et al.*, 1991, 2005; Kim *et al.*, 2009).

Others

The level and stability of the CF_1CF_o ATP synthase is strongly reduced in the *alb4* mutant (Benz *et al.*, 2009), decreasing the degree of appression in grana stacks (Gerdes *et al.*, 2006). Mutants affected in the assembly of the Cyt b_6f complex (Lennartz *et al.*, 2001; Dreyfuss *et al.*, 2003; Maiwald *et al.*, 2003; Xiao *et al.*, 2012) have not yet been characterized with respect to thylakoid ultrastructure. Nevertheless, ultrastructural data are available for a mutant with markedly reduced levels of Cyt b_6f (Manara *et al.*, 2014). However, these lines show normal thylakoid formation, indicating that the Cyt b_6f complex does not play a significant role in the establishment of the thylakoid ultrastructure (Manara *et al.*, 2014).

Interdependency of protein and lipid supply

The composition of thylakoid membranes varies little between photosynthetically active organisms (Siegenthaler, 1998; Vigh *et al.*, 2005). Lipid membranes serve as habitats for the proteins involved, playing important roles in their stability and functionality (Mizusawa and Wada, 2012; Boudière *et al.*, 2014). Liposomes consisting of DGDG and MGDG are able to stabilize LHCII trimers, while the absence of DGDG slightly destabilizes the complex (Yang *et al.*, 2006). Interestingly, increasing MGDG content in LHCII–PSII liposomes increases the antenna cross-section and boosts photosynthetic activity (Zhou *et al.*, 2009). SQDG may be similarly involved in stabilizing PSI (Sugimoto *et al.*, 2010).

Conversely, proteins can modulate the phase behaviour of MGDG. Thus, by increasing the amounts of LHCII, the inverted hexagonal phase can be progressively transformed into ordered lamellar structures (Simidjiev *et al.*, 2000). It has been hypothesized that the amount of thylakoid-incorporated non-bilayer-forming lipid is controlled by the current state of the membrane (Garab *et al.*, 2000), such that changes in protein content and distribution cause excess MGDG to be forced into a non-bilayer phase or be recruited from there (Garab *et al.*, 2000). The existence of such a non-bilayer phase and its exchange with the membrane was shown by Krumova *et al.* (2008) (see above). Only the tight packing of proteins into the membrane is compatible with the high

concentration of MGDG, and vice versa. Consequently, the protein-rich appressed grana stacks were found to have a higher MGDG:DGDG ratio than stroma lamellae (Gounaris *et al.*, 1983, 1986), in agreement with the idea that non-bilayer-forming lipids mediate stacking (Lee, 2000).

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