The Functional Network of the Arabidopsis Plastoglobule Proteome Based on Quantitative Proteomics and Genome-Wide Coexpression Analysis1[C][W][OA]

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Plastoglobules (PGs) in chloroplasts are thylakoid-associated monolayer lipoprotein particles containing prenyl and neutral lipids and several dozen proteins mostly with unknown functions. An integrated view of the role of the PG is lacking. Here, we better define the PG proteome and provide a framework for further studies. The PG proteome from Arabidopsis thaliana leaf chloroplasts was determined by mass spectrometry of isolated PGs and quantitative comparison with the proteomes of unfractionated leaves, thylakoids, and stroma. Scanning electron microscopy showed the purity and size distribution of the isolated PGs. Compared with previous PG proteome analyses, we excluded several proteins and identified six new PG proteins, including an M48 metallopeptidase and two Absence of bc1 complex (ABC1) atypical kinases, confirmed by immunoblotting. This refined PG proteome consisted of 30 proteins, including six ABC1 kinases and seven fibrillins together comprising more than 70% of the PG protein mass. Other fibrillins were located predominantly in the stroma or thylakoid and not in PGs; we discovered that this partitioning can be predicted by their isoelectric point and hydrophobicity. A genome-wide coexpression network for the PG genes was then constructed from mRNA expression data. This revealed a modular network with four distinct modules that each contained at least one ABC1K and/or fibrillin gene. Each module showed clear enrichment in specific functions, including chlorophyll degradation/senescence, isoprenoid biosynthesis, plastid proteolysis, and redox regulators and phosphoregulators of electron flow. We propose a new testable model for the PGs, in which sets of genes are associated with specific PG functions.

1 This work was supported by the National Institutes of Health (grant no. 5T32GM008500 to P.K.L.). Part of this work was carried out using the resources of the Computational Biology Service Unit of Cornell University, which is partially funded by the Microsoft Corporation.

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PGs appear to play a role in chloroplast development, senescence, and stress defense. Their shrinking and swelling in response to (a)biotic stresses and during developmental transitions, as well as in plastid biogenesis mutants, are well documented (Gaude et al., 2007; Simkin et al., 2007; Singh et al., 2010; Zhang et al., 2010). Recent results suggest that PGs are involved in active channeling of hydrophobic metabolites between the thylakoid and PG, permitted by the contiguous association of the two structures (Austin et al., 2006; Gaude et al., 2007). In particular during various abiotic stresses (e.g. nitrogen starvation, drought, or light stress), but also during senescence, components of thylakoid degradation, such as fatty acids and phytol tails from chlorophyll, are channeled into the PGs, likely accounting for the massive swelling. Within the PG, several of the observed proteins likely play a role in the recycling of such thylakoid catabolites, in addition to a role in the synthesis of isoprenyl lipids such as tocopherol and plastoquinone (Vidi et al., 2006; Ytterberg et al., 2006).

Despite the recent progress in PG analysis, it remains unclear how the PGs fit into plastid metabolism and chloroplast homeostasis, mostly because the functions for many PG-localized proteins are unknown. Key questions about PGs are as follows. (1) What determines and controls their size, shape, and content? (2) How are proteins recruited to the PG proteome, and how does the PG proteome change in response to changes in developmental state or (a)biotic conditions? (3) What are the functions of the PG proteins, and how are they related to each other? This study aims to provide a better framework to help answer these questions by defining a functional network.

We first examined the quantitative protein composition of PGs isolated from leaves subjected to 5 d of increased light intensity (500 μmol photons m$^{-2}$ s$^{-1}$) and compared this quantitatively against proteomics data sets of leaf, thylakoid, and stroma preparations to identify proteins specifically enriched in the PG. Because we used a far more sensitive mass spectrometer than were used in previous PG proteome analyses (Vidi et al., 2006; Ytterberg et al., 2006), combined with both in-gel and in-solution digestions, we expected to discover more low-abundance members of the PG proteome. Indeed, we identified a number of new PG proteins, including an M48 metallopeptidase (M48), two additional ABC1K proteins, and a senescence-associated gene (SAG) protein. The surprising localization of M48, as well as two ABC1K proteins, to PGs was confirmed by immunoblotting.

Transcripts or proteins involved in related biological pathways or complexes often accumulate simultaneously. Therefore, coexpression often implies the presence of functional or physical linkages between genes or proteins, allowing for the identification of new components of processes or protein complexes. Indeed, coexpression analysis has been used extensively in plant biology to identify putative protein functions and to determine physical or functional connections between proteins (Cartieaux et al., 2003; Rohde et al., 2004; Biehl et al., 2005; Vanderauwera et al., 2005; DalCorso et al., 2008; Sawada et al., 2009; Takabayashi et al., 2009; Bischoff et al., 2010; Fu and Xue, 2010; Ozaki et al., 2010; Lin et al., 2011).

Here, we employed such a transcriptional genome-wide coexpression analysis, using the core PG proteome as input, to provide a better framework for PG functions and to associate PG proteins to functional activities in Arabidopsis (Arabidopsis thaliana). This identified a coexpression network with four modules, each with a specific set of enriched functions, including plastid proteases, redox regulators, cyclic electron flow components, and genes encoding for a specific subset of proteins involved in plastid prenyl lipid metabolism. Specific ABC1K proteins and FBNs were centrally positioned in different modules within the network. This study better defines the core PG proteome and its functions in leaves. We propose a new conceptual model for the PGs, suggest a parallel to lipid rafts, and provide an intellectual and practical framework for further analysis.

RESULTS

Size Distribution, Extractability, Coalescence, and Purity of Isolated PGs from Light-Shifted Arabidopsis Leaves

As a starting material for our study, we grew Arabidopsis plants on soil for 2.5 weeks at 120 μmol photons m$^{-2}$ s$^{-1}$ and transferred them to 520 μmol m$^{-2}$ s$^{-1}$ for 5 d. The higher light intensity increased PG volume and yield and made them more amenable for experimental analysis (Ytterberg et al., 2006). The mild light stress treatment accelerated vegetative growth, increased anthocyanin accumulation in the leaves, and resulted in only minor visible damage at the edges of the oldest leaves but no damage to younger leaves (Supplemental Fig. S1).

Isolation of PGs from the Arabidopsis leaf rosettes was performed by sonication of isolated thylakoid membranes followed by flotation density centrifugation (Ytterberg et al., 2006). The enrichment of PGs was confirmed by immunodetection of the VTE1 protein (Fig. 1A), known to be uniquely localized in the PG as determined by yellow fluorescent protein localization and immunogold labeling (Vidi et al., 2006), immunoelectron tomography (Austin et al., 2006), and proteomics (Vidi et al., 2006; Ytterberg et al., 2006). Densitometric analysis of the immunoblots indicated a more than 400-fold enrichment of VTE1 in the PG preparations compared with the original thylakoid membranes (the starting material). We also measured an approximately 4-fold depletion of VTE1 in thylakoids following sonication, indicating that approximately 75% of the PG material is extracted from the thylakoids by sonication (Fig. 1B). Hence, our results demonstrate that the isolated PGs were highly enriched for PG particles and that the majority of the PGs were successfully extracted from the thylakoid membrane.

Transmission electron microscopy (TEM) of PGs in vivo demonstrated a broad size distribution, even
within the same chloroplast (Austin et al., 2006); however, the relationship between PG size and the PG proteome is not known. For a correct and meaningful quantitative and qualitative analysis of the PG proteome, therefore, it was critical to extract PGs representing the entire in vivo population, without bias for size or other (unknown) properties, while keeping contamination from thylakoids and other compartments to a minimum. Therefore, we compared the size distribution of the extracted PG particles with the in vivo size distribution. TEM of the leaf tissue showed a broad distribution of PG sizes, with diameters ranging from approximately 175 nm to approximately 600 nm, and peaking between 250 and 350 nm (Fig. 1, C and D). PG preparations were analyzed by scanning electron microscopy (SEM) and also showed a broad size distribution from approximately 50 nm to approximately 600 nm, peaking between 100 and 250 nm (Fig. 1, C and E). This demonstrated that PGs of all physiologically relevant sizes were extracted efficiently, with a small bias to smaller particles. Interestingly, the micrographs of PG preparations sometimes showed PGs in grape-like clusters, similar to those described in TEM micrographs of leaves (Rey et al., 2000; Austin et al., 2006; Simkin et al., 2007; Zbierzak et al., 2010; Supplemental Fig. S2, A and B). Despite the clustering found in the preparations, each PG clearly maintained its individual structure, and they did not coalesce. Apparently, component(s) at the PG-solution interface act to maintain PG structural integrity and are extractable with the PGs, likely a FBN coat surrounding the PG periphery. Evidence of minor amounts of thylakoid membrane fragments was also found in the micrographs. While the isolated PGs demonstrated remarkably smooth surfaces, SEM also showed infrequent amorphous structures generally attached to PGs (Supplemental Fig. S2C). The size of these structures, the presence of attached PGs, and their amorphous shape suggested that they are thylakoid

Figure 1. PG extraction and purification efficiency. A, Immunoblot of a thylakoid sample prior to sonication and purified PG fractions. Proteins were separated by one-dimensional SDS-PAGE and probed with antibody against the PG marker protein VTE1. 1X corresponds to 2.2 μg of protein. B, Immunoblot of thylakoid samples prior to sonication and after sonication for removal of PGs. A Ponceau stain of protein is included as a loading control. 1X corresponds to 10 μg of protein. C, Histogram illustrating the distribution of PG diameters from TEM of chloroplasts in mature leaf tissue and SEM of purified PG preparations. n ≥ 263 measurements. D, TEM of leaf chloroplast of Arabidopsis (Col-0), representative of the time point PG preparations were made. A photograph of a typical light-stressed Arabidopsis rosette plant is shown in the inset. PGs in the micrograph, marked by white arrows, appear as gray circles with black periphery or, less frequently, as solid black circles. E, SEM of Arabidopsis (Col-0) purified PGs, demonstrating the efficient isolation of PGs with varying diameters. Bars in D and E = 500 nm. [See online article for color version of this figure.]
membrane fragments. Importantly, these amorphous structures were far less abundant than the PGs, indicating high PG purity, which was further confirmed by the proteomics experiments (see below). Summarizing, our results demonstrate that more than approximately 75% of the PGs, from all physiologically relevant sizes, were successfully extracted from the thylakoid membrane into highly enriched PG preparations.

Improved Coverage and Quantification of the PG Proteome

The first comprehensive PG proteome analyses were carried out using a quadrupole time of flight mass spectrometer (Ytterberg et al., 2006) or a LCQ Deca XP ion trap mass spectrometer (Vidi et al., 2006). Recent improvements in the sensitivity, mass accuracy, and speed of mass spectrometers have enabled the detection of lower abundance proteins in complex mixtures and also facilitated mass spectrometry-based label-free proteome quantification using spectral counting (Bantscheff et al., 2007; Mann and Kelleher, 2008; Domon and Aebersold, 2010). Thus, a much more sensitive and quantitative analysis of the PG proteome should now be possible. The spectral counting technique is based on the observation that the number of successful tandem mass spectrometry (MS/MS) acquisitions of peptides coming from a protein shows a positive and linear correlation to the relative concentration of this protein in the studied sample (Liu et al., 2004; Old et al., 2005; Zybailov et al., 2005; Sandhu et al., 2008). Spectral counting is particularly effective to detect large quantitative differences, as expected in our study, where we compare (sub)cellular or suborganelar fractions that are very different in function and composition. We previously optimized the spectral counting (SPC) workflow and tested it for Arabidopsis and maize (Zea mays) organelles, cell types and complexes (Zybailov et al., 2008; Friso et al., 2010; Majeran et al., 2010; Oliinares et al., 2011). The relative normalized abundance (relative mass contribution) of each protein within each sample, NadjSPC, was calculated from the number of adjusted matched MS/MS spectra (adjSPC), normalized to the total adjSPC per sample, as defined previously (Friso et al., 2010). Thus, a protein with NadjSPC = 0.01 contributes approximately 1% of the protein mass of the analyzed sample. As a general rule, the accuracy of quantification improves with the number of adjSPC per protein.

Here, we employed an LTQ-Orbitrap mass spectrometer (Hu et al., 2005) coupled to a nano-liquid chromatography (nanoLC) system to search for additional, more low-abundance proteins located in the PG. Moreover, we reevaluated previous assignments of proteins to the PG (Vidi et al., 2006; Ytterberg et al., 2006) based on quantitative comparative proteome information. Using three independent PG preparations, the PG proteome was analyzed in two different ways: (1) PG proteins were separated by SDS-PAGE, each lane cut in five slices, and in-gel digested with trypsin, and (2) unfractionated PGs were delipidized and digested in-solution with trypsin. These protein digests were then analyzed by MS/MS in the LTQ-Orbitrap (Fig. 2A). The rationale for using these two different protein extraction/separation methods was to (1) maximize the detection of low-abundance proteins, (2) increase the robustness of protein quantification, and (3) improve protein sequence coverage. Proteins identified by only a single peptide sequence, irrespective of posttranslational modification or charge state, were discarded to increase the robustness of the analysis and avoid any false-positive protein identification; these proteins represented less than 1% of the protein mass in PGs.

Defining the Core PG Proteome

The combined proteome analysis identified 234 proteins, with 129 identified by both in-solution and in-gel workflows and six or 99 proteins identified in only the in-solution or in-gel digestion, respectively (Fig. 2A; for details, see Supplemental Table S1). The 129 proteins identified by both methods represented approximately 99% of the PG protein mass, showing that only the least abundant proteins were not identified by both methods. The in-solution and in-gel methods showed a good correlation for the relative protein abundance for proteins with abundance of greater than 0.001 (i.e. proteins that each represent more than 0.1% of the protein mass of the PGs; Fig. 2B, gray area). Protein sequence coverage was on average 26% for the in-gel method and 16% for the in-solution method; this increased to 37% and 27%, respectively, if we only considered the 129 proteins identified by both methods. The correlation of the average NadjSPC values (combining both in-gel and in-solution data) between the three biological replicates was excellent, with pairwise correlation coefficients between 0.902 and 0.960 (Fig. 2C).

We then determined those proteins highly enriched in the PGs, hereafter named the “core” PG proteome, using the workflow as depicted in Figure 3A. The core PG proteins were distinguished from nonplastid contaminants or proteins localized primarily elsewhere in the chloroplast by comparing the abundance in PGs with their average abundance in total leaf extracts (five biological replicates with two replicates from Zybailov et al. [2009] and three from this study) and isolated thylakoid and stromal fractions (Zybailov et al., 2008). Supplemental Table S2 provides the quantitative and qualitative data about these proteomes. Furthermore, core PG proteins were required to have a minimal abundance (NadjSPC > 0.001) and can be observed in the PGs by both in-gel and in-solution methods (Fig. 3A).

For the selection of core PG proteins, we first discarded those proteins with a PG-leaf abundance ratio below 10; only 52 proteins out of the 234 proteins passed this first filter. We emphasize that this was a relatively “relaxed” minimal threshold, considering that the PG proteome represents less than 10% of the leaf proteome; however, this was already very effective to remove nonplastid...
As contaminants as well as the abundant proteins of the photosynthetic apparatus and other proteins not truly enriched in PGs. Importantly, it also removed several proteins that were earlier assigned to the PG, including fructose bisphosphate aldolase 1 and 2 (FBPA-1 and -2; Vidi et al., 2006; Ytterberg et al., 2006). Furthermore, FBN3a and FBN10 were also eliminated, because they showed PG-leaf abundance ratios of only 0.9 and 4.7, respectively (Table I; Supplemental Table S1). The relative abundance and distribution of the remaining 52 proteins between PGs, thylakoids, and stroma are displayed in Figure 3, B and C.

As a next step, we removed proteins that failed to show at least a 5-fold enrichment in the isolated PGs compared with the thylakoid (Fig. 3A). This resulted in the removal of four proteins: a DnaJ domain protein, a glutaredoxin, a protein with an unknown function (AT5G62140), and AOS (Fig. 3B). Finally, four proteins with a PG-stroma abundance ratio below 20 were discarded: these were thioredoxin M4 (Trx M4), UV-B/ozone similarly regulated protein (UOS1), an unknown protein with a DUF1350 domain, and FBPA-3 (Fig. 3B).

The remaining 44 proteins were then evaluated for abundance and frequency of identification in the PG proteome analysis (Fig. 3C). Twelve proteins with a relative abundance below NadjSPC of 0.001 (corresponding to 0.1% or less of the protein mass; Fig. 3C), or only identified by one of the methods, were discarded. (We note that none of these proteins were coexpressers of the PG core genes [see below].) Finally, we manually evaluated the remaining 32 proteins for known subcellular localization and/or function.

Figure 2. Identification of the PG proteome by in-gel and in-solution methods. A, Three independent preparations of PGs were made from leaf rosettes of Arabidopsis plants grown at 120 μmol photons m⁻² s⁻¹ and a 16-h-light/8-h-dark cycle that were shifted for 5 d to moderate light intensities (520 μmol photons m⁻² s⁻¹). Thylakoids were isolated from total leaf tissue and sonicated to release PGs. Aliquots of each PG preparation were then separated by SDS-PAGE and in-gel digested or delipidized, in-solution digested, and tip zipped. In-solution and in-gel digested peptides were analyzed with a nanoLC-LTQ-Orbitrap mass spectrometer. A total of 234 unique proteins were identified, 129 of which were identified by both experimental methods. B, Comparison of NadjSPC between each of the 234 proteins in the in-gel and in-solution methods demonstrates consistent quantification of proteins above 0.001 NadjSPCs, marked in gray. C, Comparison of protein abundance (measured as NadjSPC) between each of the three biological replicates (repl). R² = correlation coefficient, N = number of proteins. Proteins only present in one of each pair were included in the correlation analysis and were represented by a zero value when absent. [See online article for color version of this figure.]
function. Two proteins were discarded from the core proteome based on literature evidence. The extra-plastidic caleosin protein RD20 (AT2G33380) has been shown to be localized in cytosolic lipoprotein particles (Aubert et al., 2010), while a PLAT/LH2 domain protein (AT2G22170) is likely endoplasmic reticulum localized based on GFP tagging (http://gfp.stanford.edu/index.html). Thus, these proteins were removed from the final list.

Because the PGs were isolated from plants shifted for 5 d to higher light intensities (520 μmol photons m⁻² s⁻¹), we also determined and quantified the total leaf proteome of these plants (three independent replicates; Supplemental Table S2). However, using these quantitative total leaf proteome data in the workflow (Fig. 3A) did not affect the final selection of core PG proteins.

The Core PG Proteome

Table II summarizes the core PG proteome with their relative abundance (including the coefficient of variation [CV]) and enrichment as compared with other plastid compartments. The CV of protein abundance across the three biological replicates was 24%, indicating an excellent reproducibility. Twenty-three of the 30 core proteins were previously assigned to the PG (Vidi et al., 2006; Ytterberg et al., 2006), with 18 identified in both studies (Table II). VTE1 showed a PG-thylakoid ratio of 131, consistent with the high ratio determined by the immunoblot analysis (Fig. 1A), and was not detected in chloroplast stroma.

Another seven proteins were newly identified as plastoglobular, namely two ABC1 kinases (ABC1K6 and -7), a PLAT/LH2 domain protein (PLAT/LH2-1), an esterase-domain protein (Esterase1), two proteins of unknown function (Unknown-2 with DUF1350 and unknown SAG), and a metallopeptidase M48 domain protein. Six of these seven proteins are the lowest abundance proteins of the core PG proteome (Table II), explaining their previous lack of detection. Thirteen proteins previously assigned to the PG did not pass...
our filters (Table II); these were AOS, FBPA-1,2,3, FBN3a, two RNA-associated proteins (Rap38 and -41), an ATPase, WAVE3, peroxiredoxin Q (PrxQ), and three proteins of unknown function (see “Discussion”). The four most abundant proteins were all FBN proteins (FBN1a, -1b, -2, and -4) and were also found previously to be the homologs of the FBNs in red pepper (Capsicum annuum) chromoplast PGs (Ytterberg et al., 2006), suggesting that they may hold a general function in the maintenance of plastid lipid body structure. The six FBN core proteins constituted 53% of the PG proteome mass (Fig. 3D). The second most abundant class of core proteins consisted of six ABC1 kinases, together constituting 19% of the core PG proteome mass. The original ABC1K proteins, identified in Saccharomyces cerevisiae (Abc1p/Coq8p) and E. coli (UbiB), are implicated in the regulation of ubiquinone metabolism (Poon et al., 2000; Do et al., 2001). In particular, the phosphorylation of several members of the ubiquinone biosynthetic complex is dependent on Abc1p/Coq8p (Xie et al., 2011). However, the role and possible targets of the PG-localized ABC1K proteins are unknown. Carotenoid Cleavage Dioxxygenase4 (CCD4), with specificity for 8-apo-β-caroten-8’-al in Arabidopsis (Huang et al., 2009), was 3.3% of the proteome mass. The VTE1 protein, involved in tocopherol biosynthesis (Porfirova et al., 2002), was 2.6% of the proteome mass, and NAD(P)H Dehydrogenase C1 (NDC1), which reduces plastoquinone to plastoquinol and is necessary for phylloquinone synthesis (Eugeni-Piller et al., 2011), was 2.5% of the proteome mass. The M48 protein was only 0.3% of the proteome mass (Table II).

**Confirmation of PG Localization of ABC1K1, ABC1K3, and Peptidase M48 by Immunoblotting**

To further validate our quantitative proteomics analysis, we generated specific antisera against three PG core proteins, ABC1K1, ABC1K3, and the low-abundance M48 protease, because of its novelty as a potential PG-localized protease. Specific polyclonal antisera were raised against affinity-purified E. coli over-expressed domains of each of the three proteins. After confirming the specificity of the sera, we compared isolated thylakoid fractions and isolated PGs for protein abundance of M48, ABC1K1, and ABC1K3 using immunoblots. Figure 4 shows that ABC1K3, ABC1K1, and M48 were approximately 10-, 20-, and more than 50-fold enriched in isolated PGs compared with (untreated) thylakoids, in agreement with the PG-thylakoid abundance ratios of 11, 16, and 17, respectively, measured by mass spectrometry (Table II). This provides independent evidence that metallopeptidase M48 and the two ABC1K proteins are highly enriched in the PG and indicates that our mass spectrometry-based quantitative analysis does provide reliable information about the core PG proteome.

**Partitioning of the FBN Proteins between PGs and the Thylakoid or Stroma**

We identified all 12 known FBNs, as well as a FBN-like protein (AT1G18060), in our collective proteome data sets of leaves, chloroplast stroma, thylakoids, and PGs (Table I; Supplemental Tables S1 and S2). However, we assigned only seven FBNs to the PG core proteome, based on our quantitative analysis (Fig. 3A),

### Table I. Subplastid localization of fibrillin proteins and their variants

| Accession No. | Name | PG/Stroma | PG/Thylakoid | PG/Leaf | PG Core | Thylakoid/Stroma |
|---------------|------|-----------|--------------|---------|---------|-----------------|
| AT4G40420.1   | FBN1a| 176       | 34           | 170     | Yes     | 5               |
| AT4G22240.1   | FBN1b| 713       | 40           | 570     | Yes     | 18              |
| AT2G35490.1   | FBN2 | 1,188     | 59           | 151     | Yes     | 20              |
| AT3G26070.1   | FBN3a| Not in stroma | 0         | 1       | No      | Only in thylakoid |
| AT3G26080.1   | FBN3b| Not in stroma | Not in PG  | Only in thylakoid | No | Only in thylakoid |
| AT3G23400.1   | FBN4 | 121       | 32           | 79      | Yes     | 4               |
| AT5G09820.1   | FBN5 | Not in PG | 139         | Only in stroma | No | Only in stroma |
| AT5G19940.1   | FBN6 | Not in PG | Not in PG   | Not in PG | No | 15              |
| AT3G58010.1   | FBN7a| 146       | 15           | 342     | Yes     | 10              |
| AT2G42130.4   | FBN7b| 23        | 11           | 55      | Yes     | 2               |
| AT2G46910.1   | FBN8 | 434       | 29           | 388     | Yes     | 15              |
| AT4G00300.1   | FBN9 | –         | –            | Only in leaf | No | –              |
| AT1G51110.1   | FBN10| Not in stroma | 2         | 5       | No      | Only in thylakoid |
| AT1G18060.1   | FBN-like| Not in PG | Not in PG   | Not in PG | No | 23              |

| Variant | FBN7a (1–290) | Found in stroma based on GFP visualization |
| Variant | FBN7a (1–133) | Found in thylakoid based on GFP visualization |

*a*Abundance ratio based on NadjSPC in PG and other chloroplast compartments. *b*Dashes indicate that the ratio could not be determined because the protein is absent in both sample types. *c*From Vidi et al. (2007).

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because the other FBNs did not preferentially locate to PGs. Therefore, we searched for physical-chemical properties of the FBN protein family that correlated with subplastid localization. We also included two truncation products of FBN7a, FBN7a (1–133) and FBN7a (1–290), whose localizations were determined by yellow fluorescent protein tagging as localized to the stroma and PG, respectively (Vidi et al., 2007). The combination of pi and hydrophobicity, calculated as the grand average of hydropathicity (GRAVY) index, for each of the FBNs correlated surprisingly well with their relative distribution between stroma, thylakoids, and PG.

| Accession No. | Protein Name | NadjSPC | CV | Percentage Mass | PG/Thylakoid | PG/Stroma | Reference | Identification |
|---------------|--------------|---------|----|----------------|-------------|-----------|-----------|---------------|
| AT4G04020     | Fibrin 1a (FBN1a) | 0.100   | 15 | 16.1           | 34          | 176       | ×         | ×            | ×            | Previously identified |
| AT3G23400     | Fibrin 4 (FBN4)   | 0.074   | 30 | 11.9           | 32          | 121       | ×         | ×            | ×            | Previously identified |
| AT4G22240     | Fibrin 1b (FBN1b) | 0.059   | 12 | 9.6            | 40          | 713       | ×         | ×            | ×            | Previously identified |
| AT2G35490     | Fibrin 2 (FBN2)   | 0.044   | 4   | 7.1            | 59          | 1,188     | ×         | ×            | ×            | Previously identified |
| AT5G05200     | ABC1K9          | 0.032   | 8   | 5.2            | 440         | 1         | ×         | ×            | ×            | Previously identified |
| AT4G31390     | ABC1K1          | 0.028   | 8   | 4.5            | 16          | –         | –         | ×            | ×            | Previously identified |
| AT1G79600     | ABC1K3          | 0.027   | 23  | 4.3            | 11          | –         | –         | ×            | ×            | Previously identified |
| AT3G58010     | FBN7a (FBN7a)   | 0.022   | 44  | 3.5            | 15          | 146       | ×         | ×            | ×            | Previously identified |
| AT4G19170     | Carotenoid dioxygenase 4 (CCD4) | 0.021   | 25  | 3.3            | 18          | 42        | ×         | ×            | ×            | Previously identified |
| AT4G32770     | Tocopherol cyclase (VT1) | 0.016   | 5   | 2.6            | 131         | –         | ×         | ×            | ×            | Previously identified |
| AT1G54570     | Diacylglycerol acyltransferase 3 (DGAT-3) | 0.016   | 12  | 2.6            | 31          | –         | ×         | ×            | ×            | Previously identified |
| AT5G08740     | NAD(P)H dehydrogenase C1 (NDC1) | 0.015   | 8   | 2.5            | 19          | –         | ×         | ×            | ×            | Previously identified |
| AT2G42130     | Fibronectin 7b (FBN7b) | 0.013   | 45  | 2.1            | 11          | 23        | ×         | ×            | ×            | Previously identified |
| AT1G32220     | Flavin reductase-related 1 | 0.013   | 23  | 2.1            | 22          | 102       | ×         | ×            | ×            | Previously identified |
| AT4G13200     | Unknown 1        | 0.012   | 17  | 1.9            | 11          | –         | ×         | ×            | ×            | Previously identified |
| AT3G10130     | SOUL domain protein | 0.011   | 10  | 1.8            | 61          | –         | ×         | ×            | ×            | Previously identified |
| AT2G46910     | Fibronectin 8 (FBN8) | 0.011   | 12  | 1.8            | 29          | 434       | ×         | ×            | ×            | Previously identified |
| AT1G71810     | ABC1K5          | 0.011   | 12  | 1.7            | 17          | –         | ×         | ×            | ×            | Previously identified |
| AT1G78180     | Ubil methyltransferase-related 1 | 0.009   | 43  | 1.5            | 48          | –         | ×         | ×            | ×            | Previously identified |
| AT1G06690     | Aldol keto reductase | 0.009   | 35  | 1.5            | 13          | 765       | ×         | ×            | ×            | Previously identified |
| AT2G44660     | Flavin reductase-related 2 | 0.009   | 26  | 1.5            | 6           | 75        | ×         | ×            | ×            | Previously identified |
| AT2G41040     | Ubil methyltransferase-related 2 | 0.009   | 17  | 1.5            | 72          | –         | ×         | ×            | ×            | Previously identified |
| AT3G26840     | Diacylglycerol acyltransferase 4 (DGAT 4) | 0.009   | 14  | 1.4            | –           | –         | ×         | ×            | ×            | Previously identified |
| AT3G24190     | ABC1K6          | 0.016   | 14  | 2.6            | 15          | 322       | ×         | ×            | ×            | Newly identified |
| AT4G39730     | PLAT/H dehydrogenase C1 | 0.016   | 29  | 1.6            | –           | –         | ×         | ×            | ×            | Newly identified |
| AT3G43540     | Unknown 2 (DUF1350) | 0.008   | 42  | 1.3            | 14          | 80        | ×         | ×            | ×            | Newly identified |
| AT3G07700     | ABC1K7          | 0.005   | 27  | 0.8            | 37          | –         | ×         | ×            | ×            | Newly identified |
| AT1G37570     | Unknown SAG      | 0.002   | 80  | 0.4            | –           | –         | ×         | ×            | ×            | Newly identified |
| AT3G27110     | M48 protease    | 0.002   | 42  | 0.3            | 17          | –         | ×         | ×            | ×            | Newly identified |
| AT5G41120     | Esterase 1      | 0.002   | 30  | 0.3            | –           | –         | ×         | ×            | ×            | Newly identified |

*aAbundance of each PG core protein. bCoefficient of variation of the average NadjSPC across the three biological replicates. cContribution of each protein to protein mass of the PG core proteome as a percentage of total core proteome. dAbundance ratio based on NadjSPC in PG and other chloroplast compartments. 1, Vidi et al. (2006); 2, Ytterberg et al. (2006); 3, current analysis. fDashes indicate that the ratio could not be determined because the protein was not detected in either thylakoid or stromal sample type."
The FBN proteins could be placed in one of four groups: (1) strongly enriched in the PG (at least 10-fold), (2) equal enrichment between the PG and thylakoid (PG-thylakoid ratio of approximately 1), (3) strongly enriched in the thylakoid as compared with PG (more than 10-fold), and (4) stroma localized, not identified in PG or thylakoids. All seven PG-localized FBNs, as well as the truncated FBN7a (1–290), were found to display low pIs and (on average) higher hydrophobicity indices. Conversely, all four FBNs strongly enriched in the thylakoid membrane fraction displayed higher pIs and lower hydrophobicity indices. Importantly, FBN10, the only FBN with an approximately equal ratio between PG and thylakoid (PG-thylakoid ratio of 1.8) showed intermediate pI and hydrophobicity index. Finally, the stroma-localized FBN5 and FBN7a (1–133) demonstrated low pI and the lowest hydrophobicity indices of the 16 protein products. The pI and GRAVY index, however, did not predict subplastid localization of other members of the core PG proteome, likely because they have very diverse secondary structures.

A PG Coexpression Network Shows Strong, Specific Enrichment for Genes of Four Plastid Functions

Because the functions of most PG proteins are unknown and hard to predict, we employed a genome-wide transcript coexpression analysis to identify putative functions for the PG core proteins, identify potential targets for the ABC1K proteins, generate testable hypotheses, and provide a better framework for further studies. Several coexpression analysis tools have been developed and employed in plant coexpression analysis, each offering its own suite of functions and set of normalized microarray experiments (Steinhauser et al., 2004; Mutwil et al., 2008; Usadel et al., 2009). We tested and compared three different publicly available coexpression tools, MetaOmGraph (Wurtele et al., 2007), the Botany Array Resource (BAR; Toufighi et al., 2005), and the Arabidopsis Coexpression data-mining Tool (ACT; Manfield et al., 2006), for their ability to identify coexpression relationships among functionally and physically associated gene products. Using the well-studied gene family encoding for the ClpPR protease complex in plastids and mitochondria (Olinares et al., 2011) and 10 genes encoding for enzymes involved in tetrapyrrole biosynthesis, we first demonstrated that although the three software programs, MetaOmGraph, BAR, and ACT, show quantitative differences in coexpression rankings, true coexpressers were consistently found (Supplemental Text S1; Supplemental Fig. S3). We also tested to see if PG core genes preferentially expressed with other PG core genes rather than genes encoding for plastid proteins in general or with genes encoding for extraplastidic proteins. This showed that PG core genes generally preferentially coexpress with other PG genes rather than genes encoding for plastid proteins in general or with genes encoding for extraplastidic proteins. This showed that PG core genes generally preferentially coexpress with other PG genes at higher Pearson correlation coefficient (PCC; Supplemental Text S1; Supplemental Fig. S4). Importantly, these tests also showed that genes encoding for plastid proteins

Figure 4. M48 metalloprotease, ABC1K3, and ABC1K1 are enriched in the PG preparations. Immunoblots of a thylakoid sample (prior to sonication) and the PGs (subsequently extracted by sonication) illustrate enrichment levels comparable to those determined by mass spectrometry. A Ponceau stain is included for each blot as a loading control. 1 x = 10 μg. [See online article for color version of this figure.]
are clearly not coexpressed as a single group; thus, we should be able to find specific coexpression patterns for PG core proteins. The selection of test sets, procedures, and results is described in more detail in the Supplemental Text S1.

We chose to employ the MetaOmGraph program to investigate the PG coexpression network because of its user-friendly nature and validated the final results with the other two programs. We discarded from the analysis those probes measuring multiple genes to ensure that we were testing specific gene-gene coexpression relationships. The resulting set contained 21,158 Affymetrix microarray probes, including 25 of the 30 PG core genes. PG core genes FBN1a and -1b, as well as DGAT4, had to be excluded because they were not represented by unique probes (see “Discussion”), whereas the SOUL and Esterase1 genes were not represented on the microarrays.

A PG network was constructed from a genome-wide search for each of the 25 PG core genes on the Affymetrix microarray. Some of the PG genes, e.g. FBN2 and -4, aldo/keto reductase (AKred), Unknown-2, had several hundred coexpressing genes above a PCC threshold of 0.7 (or in some cases even above 0.8), whereas other genes (ABC1K7, UbiE-2, M48, DGAT3, unknown SAG) had none above that threshold. Therefore, we used the 20 strongest coexpressing genes for each PG core gene to construct a PG network, rather than applying a minimal PCC threshold. All such coexpression relationships had a PCC above 0.65, with the exception of the PLAT/LH2 domain protein and the SAG protein with unknown function. Strong negative correlations between genes can be relevant; however, negative PCC values never exceeded an absolute value of 0.67, and therefore only positive correlations became part of the PG network.

The resulting network contained 374 nodes (genes) and 500 edges (coexpression interactions; Fig. 6). Of the 374 nodes, 201 (54%) were assigned to the plastid based on experimental information (Supplemental Table S3). Interestingly, the PG core proteins differed strongly in the subcellular localization of their coexpressers. For instance, in the case of the five FBNs, NDC1, ABC1K3, and several others, 17 to 20 out of 20 of the coexpressers were plastid localized; however, core proteins VTE1, UbiE1, DGAT3, and PLAT/LH2 domain protein each had three or fewer plastid-localized coexpressers. This immediately suggests that the latter proteins are primarily posttranscriptionally regulated or that their transcriptional regulation is integrated with extraplastidic functions and needs.

To better assign functions to the PG core genes and the PG as a whole, coexpressing genes were categorized by their assigned functional category (using the MapMan bin system as the basis to organize the functions), and edges connecting to each bin were counted. Because some bins were much larger than others and thus had a much greater opportunity to be represented in the PG network, we normalized the representation of each bin by its size. As indicated in Table III, a strong enrichment was found for plastid-localized proteases (17 in total; LON, Prep1, EGY2, PtsH1, -2, -5, -8, -9, ClpR2, -R3, -P4, -P5, -P6, -S1, -C1, -D, DegP1 and -8), proteins involved in cyclic/alternative electron flow (five NDH subunits; PGR5, PGR11A and -B, PTOX, PIF5), and regulators of the light reaction state transition kinase (STN7 and phosphatase TAP38), plastid-localized isoprenoid metabolism (in particular carotenoid metabolism; PDS, ZDS, LYC-β, zeaxanthin epoxidase [ZEP]), chlorophyll degradation (pheophorbide a oxygenase [PaO]/ACD1, pheophytinase [PPH], ACD2), and plastid redox regulation (Trx-M1, -2, -4, Trx-F1, Fd-Trx reductase subunits, NADPH reductase, PrxQ, and others; Table III; Supplemental Table S3). To further substantiate the findings from the MetaOmGraph coexpression analysis, we analyzed the functional enrichment of the top 20 coexpressers using the two other software programs, BAR and ACT (Supplemental Tables S3 and S4). Clearly, the distribution of functional groups was consistent between all three programs, strengthening the significance of the MetaOmGraph analysis.

The PG Coexpression Network Shows Four Modules

The coexpression network showed that most core genes had associations with other PG genes, producing a gene expression network with four clusters of nodes, which we refer to as “modules.” Modules are parts of biological networks in which nodes are densely connected with each other but between which there are only sparse connections. Thus, within each of these modules, genes coexpress more tightly to each other than with genes outside the module (Fig. 6; Table III). The modular nature is an important property of biological networks. As will be detailed below, the four modules each showed enrichment for specific functions. The remainder of the PG core genes (FRed-1, UbiE-1, PLAT/LH2-1, and VTE1) had no or weaker associations with other PG genes (Fig. 6); moreover, they had in common that most of their coexpressers encoded for extraplastidic proteins.

Module 1, with four PG core genes (DGAT3, ABC1K7, SAG, and M48 metalloprotease), was enriched for senescence functions, in particular chlorophyll degradation (PaO and PPH) and a variety of proteases outside the plastid (including a senescence-associated Cys protease), as well as the senescence-induced Clp protease chaperone ClpD1. The two chlorophyll degradation enzymes coexpressed with ABC1K7 and SAG (Fig. 6, nodes 1 and 2), and we note that a third, more downstream enzyme (red chlorophyll catabolite reductase), was found in module 3 coexpressing with FBN4. Strikingly, only 35% of the edges in module 1 were plastid localized, compared with 71% to 95% for the other modules, consistent with the observation that senescence leads to controlled breakdown of the whole cell, and is not limited to plastids. We also point out an interesting plastid-localized putative Tyr kinase that coexpressed with both DGAT3 and ABC1K7 (Fig. 6, node 18). The role for M48 protease is completely unknown, and its coexpressers included five plastid proteins with unknown function and four plastid-localized
proteins: PPH, ClpR3, thylakoid alternative oxidase (PTOX), and a glutaredoxin thioredoxin.

The most extensive module (module 2) was centrally located in the PG network and comprised eight PG genes (ABC1K-1, -3, -6, aldo/keto reductase, NDC1, flavin reductase 2, CCD4, UbiE-2). It was particularly enriched for carotenoid metabolism enzymes (for the complete pathway and the connections to coexpressers, see Supplemental Fig. S5) and plastid proteases (22 edges); 71% of the nodes encoded for plastid proteins, indicating tight integration with plastid functions (Table III; Fig. 6). In addition to the plastid carotenoid enzymes, also upstream cytosolic solanesyl diphosphate synthase (SPS1) and its plastid isoform (SPS2; responsible for synthesizing the hydrophobic tail of plastoquinone [PQ9]) were part of this module (we note that MEP pathway enzymes are only found in module 4). Interestingly, GOLDEN2-LIKE1 transcription factor, known to coregulate the expression of a suite of nuclear photosynthetic genes (Fitter et al., 2002), was also part of this module as a coexpresser of CCD4. Within module 2, ABC1K3, AKRed, and NDC1 were particularly tightly connected, mostly through coexpressing plastid proteases. The top 20 coexpressers of ABC1K3 were almost exclusively involved in carotenoid metabolism (ZEP, PDS, ZDS, PDS1; 5, ZDS; 6, PDS1; 7, FtsH2; 8, DegP1; 9, EF-TU-Lep; 10, FdI-like; 11, Trx M1; 12, Trx M2; 13, FTR6; 14, CcdA Cryt assembly; 15, AKRed-like; 16, haloacid dehalogenase domain protein; 17, methyltransferase domain protein; 18, Tyr kinase; 19, β-glucosidase 9; 20, ZEP; 21, NAD kinase 2; 22, STN7; 23, TAP38; 24, PTOX/Immutsans; 25, NDH-N; 26, NDF1; 27, NDF2; 28, MCS; 29, CSK.

Figure 6. PG network visualization and functional enrichment. For each PG gene, the 20 strongest coexpressing genes from a genome-wide analysis by MetaOmGraph were compiled into a PG coexpression network and visualized with the Cytoscape software program using the force-directed layout algorithm. Each gene is represented by a single node. Edges, representing coexpression interactions between PG genes and coexpressed genes, are colored according to the functional annotation of the coexpressed gene. Coexpression relationships between two PG genes are indicated with red. Visualization reveals four functional modules in which coexpressed genes are enriched in specific cellular/plastidic processes. Each module is shaded in gray, and the enriched cellular processes are indicated. Six PG genes are not included in a functional module. For each, the number of plastid-targeted genes (out of 20) and potential relevant coexpressers are listed. Twenty-seven coexpressers that are located at important positions in the network, and/or that have particularly interesting functions, are marked with numbers as follows: 1, PPH; 2, PaO (or ACD1); 3, FtsH8; 4, CCD1; 5, ZDS; 6, PDS1; 7, FtsH2; 8, DegP1; 9, EF-TU-Lep; 10, FdI-like; 11, Trx M1; 12, Trx M2; 13, FTR6; 14, CcdA Cryt assembly; 15, AKRed-like; 16, haloacid dehalogenase domain protein; 17, methyltransferase domain protein; 18, Tyr kinase; 19, β-glucosidase 9; 20, ZEP; 21, NAD kinase 2; 22, STN7; 23, TAP38; 24, PTOX/Immutsans; 25, NDH-N; 26, NDF1; 27, NDF2; 28, MCS; 29, CSK.

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particularly enriched in redox regulators and “photoacclimation.” Eighty-nine percent of the edges encoded for assigned plastid proteins. The module was highly enriched for plastid redox regulators (including thioredoxin M1, -2, -4, and F1, two Fd-Trx reductase subunits, glutaredoxins) and nonlinear electron flow components (NDH, PGRL1A, PIFI) as well as several plastid proteases (ClpR2, -P5, -S, DegP1, FtsH2; Fig. 6). Also, the thylakoid phosphatase TAP38 and the gene coding for the “acclimation of photosynthesis to environment” were part of this module.

The fourth and smallest module contained PG core proteins FBN7b and Unknown-2 and was strongly enriched for proteins involved in various aspects of plastid biogenesis, including proteases, and the Calvin cycle. Remarkably, PG core protein Unknown-2 coexpressed with six different Calvin cycle genes (FBPase, FBPA, sedoheptulose-bisphosphatase, phosphoribulokinase, G3P-DH, phosphoglycerate kinase) as well as the catalytic subunit of Gly decarboxylase, critical for photorespiration. FBN7b appears to be involved in plastid/thylakoid biogenesis; among its top coexpressers are genes coding for THYLAKOID FORMATION1, vesicle-inducing protein in plastids, plastid division protein Giant Chloroplasts1, and several genes of protein synthesis, assembly, folding, and targeting. MEP enzymes IspF and HDS were also found in module 4 as coexpressers of FBN-2 and -4 and ABC1K9.

FBN8 was positioned between modules 2 and 4, and its coexpressers (all 20 were plastid localized) were enriched in plastid biogenesis, photosynthesis, and several proteins without known function. ABC1K5 connected to both modules 2 and 4, and its coexpressers (16 were plastid localized) were enriched in NDH subunits, transporters, and various unknowns.

It is important to note that only one of the genes (CF1-γ) encoding for known structural proteins of the linear electron transport chain and ATP synthase (e.g. PSI or PSII, the cyt b6f complex, or ATP synthase) coexpressed with the PG core genes. However two of the three

| Functional Group                  | Bin Sizea | Whole Networkb | Module 1b | Module 2b | Module 3b | Module 4b |
|-----------------------------------|-----------|----------------|-----------|-----------|-----------|-----------|
| Protein degradation               | 1,355     | 0.1            | 0.1       | 0.2       | 0.1       | 0.1       |
| Plastid                           | 42        | 3.3            | 1.2       | 6.5       | 3.3       | 3.6       |
| Not plastid                       | 1,313     | 0.0            | 0.1       | 0.0       | 0.0       | 0.0       |
| Core PG                           | 25        | 3.4            | 4.0       | 4.5       | 4.8       | 2.0       |
| Light reaction                    | 139       | 0.8            | 0.4       | 0.5       | 1.4       | 1.1       |
| Light stress-Lil/Sep/Ohp          | 8         | 0.5            | 0.0       | 1.6       | 0.0       | 0.0       |
| NDH dependent and independent,    | 29        | 1.5            | 1.7       | 0.4       | 2.8       | 0.0       |
| Immotans, PIFI                    |           |                |           |           |           |           |
| PSI, PSII, ATPsynt, Cytb6f, FNR,  | 94        | 0.5            | 0.0       | 0.3       | 0.9       | 1.6       |
| electron carriers (PC, Fd)        |           |                |           |           |           |           |
| Thylakoid-bound regulators,       | 5         | 3.2            | 0.0       | 5.0       | 8.0       | 0.0       |
| including kinases and phosphatases|           |                |           |           |           |           |
| Isoprenoid metabolism             | 124       | 0.7            | 0.2       | 1.6       | 0.5       | 0.4       |
| Plastid                           | 59        | 1.1            | 0.0       | 2.5       | 1.0       | 0.8       |
| Not plastid                       | 65        | 0.3            | 0.4       | 0.8       | 0.0       | 0.0       |
| Redox                             | 187       | 0.7            | 0.4       | 0.3       | 2.5       | 0.3       |
| Tetrapyrrole metabolism           | 50        | 0.5            | 2.0       | 0.0       | 0.4       | 0.0       |
| Stress                            | 690       | 0.1            | 0.1       | 0.1       | 0.0       | 0.0       |
| Miscellaneous                     | 1,274     | 0.1            | 0.2       | 0.1       | 0.1       | 0.1       |
| Protein, other                    | 1,535     | 0.1            | 0.0       | 0.1       | 0.1       | 0.1       |
| Plastid                           | 211       | 0.7            | 0.0       | 0.8       | 0.4       | 2.8       |
| Not plastid                       | 1,326     | 0.0            | 0.1       | 0.0       | 0.0       | 0.0       |
| Not assigned                      | 7,707     | 0.1            | 0.1       | 0.1       | 0.1       | 0.0       |
| Development                       | 521       | 0.1            | 0.2       | 0.1       | 0.0       | 0.0       |
| CHO metabolismc                   | 441       | 0.3            | 0.0       | 0.2       | 0.2       | 0.9       |
| Transport                         | 944       | 0.1            | 0.1       | 0.1       | 0.0       | 0.1       |
| Otherd                            | 2,128     | 0.1            | 0.1       | 0.1       | 0.0       | 0.0       |
| Signaling                         | 1,048     | 0.0            | 0.0       | 0.0       | 0.0       | 0.0       |
| Lipid metabolism                 | 331       | 0.0            | 0.1       | 0.0       | 0.0       | 0.0       |
| DNA/RNA                          | 2,659     | 0.0            | 0.0       | 0.0       | 0.0       | 0.0       |

aNumber of genes (represented by a single probe spot on the 22K Affymetrix microarray chip) in each bin. bNumber of edges per bin, normalized for bin size and normalized for number of PG core genes per module. Values in boldface are enriched functions. cIncludes major and minor carbohydrate metabolism, gluconeogenesis, glycolysis, tricarboxylic acid cycle, C1 metabolism, fermentation, oxidative pentose phosphate pathway, Calvin cycle, and all other dark reactions. dIncludes cofactor and vitamin metabolism, metal handling, xenobiotics, amino acid metabolism, nucleotide metabolism, cytoskeleton, mitochondrial electron transport, cell wall, cell, cell division, cell cycle, nitrogen metabolism, photorespiration, polyamine metabolism, sulfur assimilation, secondary metabolism (excluding isoprenoids/tetrapyrrole), and hormone metabolism.
known genes that control state transitions (both STN7 and TAF53) and several structural components of cyclic (NDH and PGR components) or alternative (PTOX) electron flow were part of the coexpression network. This suggests that the PG function is tightly integrated with cyclic electron flow or the balance between PSI and PSII activity. We note that four lumenal OEC-23-like proteins with unknown function, as well as two unusual low-abundance LHCI-5 and LHCI-7 proteins (AT1G45474 and AT1G76570), were found as coexpressers, suggesting that they have functions related to optimization of the light reactions under stress conditions. Indeed, LHCI-5 is a component of the PSI-NDH supercomplex and necessary for its formation and stability, particularly under times of stress (Peng and Shikanai, 2011). The coexpression profile was found to be very similar to that of NPQ4 (PsbS) and LIL3 involved in chlorophyll or tocopherol biosynthesis (Klimmek et al., 2006). LHCI-7 was found to be up-regulated in response to light stress (Alboresi et al., 2011) and blue or far-red light treatment (Sawchuk et al., 2008).

Three of the PG core proteins were not placed in the coexpression network because they were on the same probe (on the microarrays) as a close homolog. Indeed, evaluation of DGAT4 on the same probe as a closely related nonplastid homolog (AT3G26820) showed that the top 20 coexpressers were mostly involved with senescence but not in the plastid, and they did not connect well to the PG network. However, homologs FBN1a and b, both PG core proteins and together on a single probe spot, connected tightly in the network, with coexpression with core protein ABC1K3 and its coexpressors RbcX and FtsH8, and also coexpressing with RD20 and POT, both coexpressers of ABC1K7. Thus, FBN1a/b is located in the network between module 1 and module 2.

Experimental Verification of the Coexpression Network

The coexpression network suggested that a subset of the PG-localized proteins (module 1) is involved in senescence responses. Therefore, we tested for five genes (ACD1, PPH, DGAT3, ABC1K7, and Metal Chelating Substance [MCS]) from module 1 whether transcript accumulation was indeed up-regulated during natural senescence. As a control, we also tested two genes from module 4 (ABC1K9 and FBN4) that have no obvious senescence association in the network. To that end, Arabidopsis rosette leaves were harvested during bolting and flowering, during which leaves show increased visual signs of natural leaf senescence. Because PGs are found to accumulate fatty acid phytol esters, with the phytol generated by breakdown of chlorophyll (Gaude et al., 2007), the uncharacterized esterase identified in our PG core proteome is an excellent candidate enzyme for the esterification of free phytol at the PG. The flux into phytol esterification would be expected to be highest during senescence-induced chlorophyll degradation, and we thus tested whether expression of the esterase, which is not represented on the 22K microarrays, is also senescence-induced. Reverse transcription-PCR experiments were then carried out on three biological replicates (Fig. 7). Indeed, expression of the five genes from the senescence-associated module 1 and the esterase, but not the two genes from module 4, is induced by senescence, thus providing support for our coexpression network and our hypothesized functions for the esterase and MCS gene products.

DISCUSSION

The Core PG Proteome

We identified and quantified proteins highly enriched in the thylakoid-associated PG as compared with other subplastid locations (the core PG proteome), and we associated key functions to the PG using genome-wide coexpression network analyses. PG localization for core proteins ABC1K1 and -3 and M48 metalloprotease was confirmed by immunodetection. We determined that 13 proteins, previously assigned to the PG, were not particularly enriched in the PGs; instead, they appeared primarily localized in the stroma or thylakoid membranes. Indeed, eight of these were not found in the coexpression network, whereas the others had only a single connection to a PG core gene (RAP38 and FBPA-2 to Unknown-2, RAP41 to ABCK1, FBPA-1 to FRed2, PrxQ to FBN7b). Importantly, we extended the known PG proteome with six new proteins of low abundance, including M48 protease. These new PG proteins were well integrated in the coexpression network, providing further support for their PG localization and function.

Chloroplast Protein Distribution and Recruitment of Proteins to PGs

It is not known how proteins are recruited to the PGs. This could occur by de novo synthesis and direct targeting to PGs. Alternatively, proteins could be recruited from other locations (e.g. stroma or thylakoid) to the PG through (ir)reversible protein modifications or through changes in the lipid/metabolite composition of the PGs. We can draw a parallel with the recruitment of proteins to lipid rafts, which are membrane microdomains with a distinct lipid and protein composition (Simon-Plas et al., 2011). Such lipid rafts in plant plasma membranes have emerged as a regulatory mechanism governing physiological responses, in particular with a role as signal transduction platforms during stress. In the lipid rafts, proteins (typically low abundance) are brought physically together such that they form functional modules to carry out specific functions. Because the PG can rapidly change in size and number in response to (a)biotic stresses, it seems likely that both de novo synthesis as well as the recruitment of existing proteins could occur. In the latter case, proteins should show dual localization between PGs and other plastid compartments (as is the case for most PG core proteins), whereas the first group should be exclusively localized...
to PGs with changing cellular concentrations dependent on PG size and abundance.

Indeed, we determined that some of the PG core proteins showed a far stronger enrichment to the PGs (e.g., ABC1K9 and VTE1) than others (Table II). Dynamic changes in localization have been reported for some FBN proteins, but the mechanisms are unknown. The FBN1a homolog in tobacco (Nicotiana tabacum) and pepper was distributed primarily in the stroma under optimal conditions but redistributed to the thylakoid (including PGs) in response to light or drought stress (Rey et al., 2000; Simkin et al., 2007). Based on our experimental data and the empirical relationship between the physicochemical parameters of FBN proteins and their distribution between PG and thylakoid preparations (Fig. 5), we suggest that FBN10 is a good example of a protein dual localized between PGs and the stroma-exposed thylakoid surface. The direct membrane continuity between the thylakoid and PG, demonstrated elegantly by Austin et al. (2006), could permit the movement of proteins between these two membrane systems. How unique protein compositions are maintained between them has not been demonstrated conclusively; but it is likely that protein modifications such as (de)phosphorylation, prenylation, or redox regulation may alter the distribution.

FBPA1, -2, and -3 were previously identified in isolated PGs (Vidi et al., 2006; Ytterberg et al., 2006), and transient expression of GFP-tagged FBPA1 and -2 (AT2G21330 and AT4G38970) in isolated protoplasts demonstrated an association to PGs (Vidi et al., 2006). Our current quantitative, comparative analysis clearly demonstrated that these abundant FBPAs mostly localized to the stroma, with only a small portion found in isolated PGs. We suggest that small amounts of these FBPAs could be recruited to the PG (but their function is not understood) and that the concentration effect at the PG surface, compared with the diffuse signal from the much larger stroma volume, explains the apparent, more exclusive PG localization observed by GFP tagging.

Our evidence that a significant number of genes involved in plastid isoprenoid/carotenoid accumulation are transcriptionally coordinated with genes encoding for PG proteins suggests that at least a subset of PG proteins are synthesized de novo concurrent with isoprenoid metabolism. Consistent with this notion, the expression of Erwinia uredovora phytoene desaturase in potato (Solanum tuberosum) tuber enhanced carotenoid metabolism while simultaneously increasing transcript levels of the FBN homolog CDSP34 (Ducruex et al., 2005).

The PG Coexpression Network Suggests Several PG Functions: An Integrated Model

PGs are believed to function in chloroplast development, stress responses, lipid metabolism, and senescence, including chlorophyll degradation (for review, see Bréhelin et al., 2007; Bréhelin and Kessler, 2008; Singh and McNellis, 2011). Because PGs function in so many diverse processes, and because most PG proteins have no known function, it has been difficult to obtain an integrated view of the role of the PG and assign PG core proteins to specific tasks or processes. To provide a framework for PG function, and to associate putative functions or processes to PG proteins with unknown functions, we determined the coexpression network based on the 20 most tightly associated coexpressers for each PG core gene. This resulted in four modules, each with a clear enrichment for specific functions, indicating that subsets of the PG core proteins work together to carry out specific roles.

Based on the core PG proteome information and modular structure of the coexpression network, as well as extensive published information, we created a summarizing model that integrates PG functions with chloroplast photosynthesis and metabolism, chloroplast responses to abiotic stress, and senescence (Fig. 8). A detailed description explaining the various pathways and processes is provided in the figure legends. In the remainder of this “Discussion,” we will briefly summarize suggested PG functions and summarize our conclusions for FBNs and ABC1K proteins.

Function 1. The Role of PG during Leaf Senescence

During senescence, the thylakoid membrane is dismantled, resulting in the free monogalactosyl and digalactosyl glycerols and free fatty acids. These can

Figure 7. Gene expression of eight selected genes in Arabidopsis leaves during natural leaf senescence determined by reverse transcription-PCR. Transcript accumulation is shown for five genes from module 1 (ACD1, PPH, DGAT3, ABC1K7, and MCS), two genes from module 4 (ABC1K9 and FBN4), and the uncharacterized esterase (AT5G41120), which was not on the microarray experiments and therefore could not be incorporated into the coexpression network. ACTIN2 was used as an internal loading control. Leaf tissue was selected from five time points during the course of natural leaf senescence: 1 = leaf rosette from plants beginning to bolt; 2 = leaf rosette from plants beginning to flower; 3 = senescing leaf approximately 10% chlorotic; 4 = senescing leaf approximately 50% chlorotic; 5 = senescing leaf approximately 50% chlorotic, 1 week later in senescence. The experiment was carried out in three independent replicates with similar results; data for one of the replicates are shown.
be used as substrates by AOS for the production of jasmonic acid or stored as triacylglycerol by DGAT3/4 (Fig. 8). The PG likely serves as a transient storage space for these glycerols and fatty acids. Concomitant with the breakdown of the thylakoid bilayer, thylakoid protein complexes and associated pigments such as chlorophylls are degraded. The first steps in chlorophyll degradation are the removal of Mg\(^{2+}\) from the porphyrin ring by an unknown protein, tentatively named MCS, and cleavage of the phytol from the porphyrin ring by PPH, one of the coexpressers in module 1 (Fig. 8; Hörtensteiner and Kräutler, 2011). The toxic free phytol has been shown to become esterified to fatty acid and deposited in the PG (Gaude et al., 2007). We speculate that the PG-localized esterase could be responsible for this esterification, and its transcript levels did increase during natural senescence; experiments are now under way to test this hypothesis. AT5G17450, a coexpresser in senescence module 1, is a candidate for MCS because it has a metal-binding domain (HMA). This protein must have a very low abundance, as it has not been identified by proteomics.

Function 2. PG Function in Isoprenoid Metabolism

The largest set of PG core proteins and their coexpressers were involved with plastid isoprenoid metabolism, in particular carotenoid metabolism, including two PDS isoforms, ZDS, LYC-β, β-OHase, ZEP, and CCD1 (for the complete isoprenoid pathway and the projected coexpressers, see Supplemental Fig. S5). Particularly interesting was the finding that PG genes coexpressed with PDS and ZDS, as these enzymes transfer electrons from their carotenoid substrate to the plastoquinone pool, a major component of the PG metabolome (Bailey and Whyborn, 1963; Greenwood et al., 1963; Tevini and Steinmuller, 1985). The other isoprenoid genes, upstream of the carotenoid biosynthetic pathway, were both isoforms of solanesyl diphosphate synthase (SDS1 and -2) as well as MDS and HDS of the MEP pathway. Plastid-localized SDS2 is responsible for synthesizing the hydrophobic tail of PQ-9. In particular ABC1K3, and to a lesser degree ABC1K1, was part of the network of these isoprenoid genes. The surprising linkage within the PG network of isoprenoid metabolism to plastid proteolysis can be easiest explained by the observation that these plastid proteases, in particular the thylakoid FtsH complex, are “household” proteases, thus removing proteins that are unwanted or damaged, followed by the release of chlorophylls and carotenoids. Indeed, it has been demonstrated that carotenoids and chlorophyll a are continuously synthesized and degraded in photosynthesizing leaves and indicate distinct acclimatory responses of their turnover to changing irradiance (Beisel et al., 2010).

The abundant PG compounds \(\alpha\)-tocopherol, PQ-9, and plastochromanol-8 are effective antioxidants in vivo (Havaux et al., 2005; Szymańska and Kruk, 2008, 2010). All three compounds are known to accumulate in response to light stress, most of which is likely accumulating in the PG (Vidi et al., 2006; Zbierzak et al., 2010). Within the PG, these antioxidants can rereduce the sequestered oxidized lipids. As part of module 2, four enzymes with (putative) oxidoreductase activity are present in the PG (NDC1, aldo/keto reductase, and flavin reductase 1 and 2), which (may) act in the regeneration of spent antioxidants in the PG by reducing carboxyl groups. Consistent with this possibility, vitamin K epoxide/naphthoquinone reductase was found to specifically reduce phylloquinone and menaquinone to their quinol forms in vitro (Furt et al., 2010). NDC1 has recently been demonstrated to display NAD (P)H reductase activity toward a PQ-9 analog, decyl-PQ (Eugenii-Piller et al., 2011). We speculate that the four PG oxidoreductases are active in rereducing oxidized lipophilic compounds sequestered in the PG, thereby affecting the thylakoid redox state (see function 3 below).

Function 3. Contribution of PGs in the Optimization of Photosynthesis, Light Acclimation, and Repair

Among the predominant genes coexpressing with members of the PG are the state transition kinase STN7 involved in balancing PSI and PSII activity, structural components of cyclic PSI and PSII activity, structural components, and chloroplast metabolism in general (Livingston et al., 2010a, 2010b). Interestingly, the PG coexpression network also included the chloroplast sensor kinase CSK (AT1G67840), coexpressing in module 3 with both ABC1K9 and FB2N2, and many components of the chloroplast redox network. CSK was recently shown to be involved in the redox-coupled transcriptional regulation of chloroplast genes (Puthiyaveetil et al., 2008). Furthermore, ZEP, involved in the reversible conversion of zeaxanthin to violaxanthin (via antheroxanthin) within the xanthophyll cycle, was centrally located in the gene expression network with connections to ABC1K3, ABC1K6, and CCD4. We speculate that ZEP activity may be regulated by one of these ABC1K proteins. Consistently, it was suggested that ZEP activity is controlled by a direct, as yet unidentified, modification that does not involve the state transition kinases (Reinhold et al., 2008). There is some indirect evidence that the phosphorylation of ZEP significantly impedes its in vivo activity (Xu et al., 1999). Collectively, it appears that the PG plays a key role in the short-term regulation and balancing of photosynthetic activities. Perhaps surprising, none of the well-known enzymes involved in the detoxification of soluble reactive oxygen species (superoxide and hydrogen peroxide) such as superoxide dismutases, or...
Figure 8. A model for PG function in plastid metabolism and short- and long-term photo response and adaptation. The physical connectivity of the PG and thylakoid permit extensive exchange of metabolites between the two subcompartments and possibly also facilitate the recruitment of proteins from the thylakoid-stroma-exposed surface to the PG. During times of high lipid or protein turnover (such as senescence, stress, or plastid biogenesis), the role of the PG becomes especially pertinent. We illustrate here some of the proposed functions of the PG in these processes. Turnover of galactolipids by DAD1-like acylhydrolases will release free fatty acids transported to the PG, where they can (1) be incorporated into triacylglycerol (TAG) by diacyl glycerol acyl transferase 3 and 4 (DGAT3/4), (2) enter the jasmonic acid synthesis pathway, in the case of linolenic acid (18:3), or (3) be esterified to free phytol into fatty acid phytyl esters (FAPEs) during concurrent chlorophyll degradation. Alternatively, the free phytol can be recycled for incorporation into tocopherols by two subsequent phytol kinases, the first of which has been identified (VTE5; Valentin et al., 2006). During chlorophyll degradation, the tetrapyrrole head group is captured by the PG-localized SOUL/heme-binding protein (SOUL/HBP) and delivered for further degradation to the stroma. We predict that the four PG oxidoreductases (NDC1, AKRed, and FRed1 and -2) are active in rereducing oxidized lipophilic compounds sequestered in the PG. Supporting this, NDC1 has recently been demonstrated to display NAD(P)H reductase activity toward a PQ-9 analog (decyl-PQ; Eugeni-Piller et al., 2011). We expect that NDC1 and the other PG oxidoreductases are responsible for the regeneration of oxidized PG quinones following reactive oxygen species (ROS) scavenging. PQ-9 is expected to be exchanged between the PG and thylakoid. Selective uptake of reduced (or oxidized) PQ-9 would permit a powerful control over the redox state of PQ-9 in the thylakoid and thus over a number of processes regulated by the PQ-9 redox state, including photosynthetic electron flow, retrograde signaling, carotenoid desaturation, and light-harvesting complex II (LHCII) state transition. The presence of the carotenoid cleavage dioxygenase 4 (CCD4) suggests the presence of carotenoid catabolism at the PG. Carotenoids released from the photosynthetic apparatus (photosystems and light-harvesting complexes) can be directed to the PG by FBN4 (or other FBNs) for degradation by CCD4. ABC1K9, positioned as a hub in module 4 (Fig. 6), is regulating the localization or function of FBNs and Trxs as well as components of the cyclic electron flow apparatus (NDH and/or PGR5 dependent). FBNs will be controlling the size of PGs (dashed arrow), while Trxs will control Calvin cycle activity to match the supply of reducing power produced from photosynthesis. Increased Calvin cycle activity will create additional demand for ATP that can be met by up-regulated cyclic electron flow (CEF), either NDH or PGR5 dependent (Livingston et al., 2010a, 2010b). Metabolites are enclosed in gray boxes, PG-localized proteins are marked in red, and coexpressers are marked in blue. Abbreviations used and not already defined are as follows: zeaxanthin epoxidase (ZEP), state transition kinase (STN7), phytoene desaturase (PDS), 9-carotene desaturase (ZDS), 9,13-hydroperoxy-octadecatrienoic acid (9,13-HPOT), allene oxide synthase (AOS), pheophytinase (PPH), metal-chelating substance (MCS*) possibly represented by AT5G17450, pheophorbide a (pheide a), 12-oxo-phytodienoic acid (OPDA), polyunsaturated fatty acids (PUFA), abscisic acid (ABA), red chlorophyll catabolite (RCC), red chlorophyll catabolite reductase (RCCR), and primary fluorescent chlorophyll catabolite (pFCC).
thylakoid and stromal APX, were found in the PG coexpression network.

**FBNs and ABC1K Proteins: Distribution, Functions, and Targets**

The seven PG-localized FBNs (1a, 1b, 2, 4, 7a, 7b, 8) and the six ABC1K proteins constituted more than 70% of the PG protein mass. These six ABC1K proteins are expected to act as enzyme regulators, likely via phosphorylation (Do et al., 2001), and the notion of a regulatory function is strengthened by their position as hubs in the PG network (Fig. 8). Their PG localization suggests that they are regulating enzymes that locate, at least transiently, to the PG; the coexpression network provides potential target genes that should now be experimentally tested. We note that the ABC1K homolog AT5G64940, which we annotated as ABC1K8, was never found in the PG or in the coexpression network. ABC1K8 was identified as a chloroplast inner envelope protein, and reduced expression resulted in increased sensitivity toward oxidative stress and high light (Jasinski et al., 2008). The FBN proteins are suggested to primarily function as structural proteins, likely determining PG size, some involved in the adaptation to environmental stress and others possibly influencing metabolite and protein content. Information about their possible functions is summarized in a recent review (Singh and McNellis, 2011). The seven PG-localized FBNs were distributed across the coexpression network, thus providing further suggestions for functions.

**MATERIALS AND METHODS**

**Preparation of PG and Thylakoid Material**

The PG isolation method was adapted from Itterberg et al. (2006). For each PG preparation, two flats (approximately 150 individuals) of Arabidopsis (Arabidopsis thaliana; ecotype Columbia [Col-0]) were grown on soil for 2.5 weeks under 120 μmol photons m⁻² s⁻¹ with a 16-h photoperiod. Plants were then transferred to 520 μmol photons m⁻² s⁻¹ during the dark period. In the morning of day 6, leaf tissue was harvested and homogenized in grinding buffer (50 mM HEPES-KOH, pH 8.0, 5 mM MgCl₂, 100 mM sorbitol, 5 mM ascorbic acid, 5 mM reduced Cys, and 0.05% [w/v] bovine serum albumin). Homogenate was filtered through four layers of 20-μm Miracloth, and thylakoid membranes were pelleted by centrifugation for 6 min at 1,800g. Thylakoid pellets were washed once in 4 volumes of grinding buffer and resuspended in medium R (50 mM HEPES-KOH, pH 8.0, 5 mM MgCl₂, and a cocktail of protease inhibitors) containing 0.2 mM Suc. An aliquot of resuspended thylakoid material was stored at −80°C to be used as the presonicated thylakoid fraction. The remainder was sonicated four times for 5 s each at output power of 23 W (Fisher Scientific; sonic dismembrator model 100B), returning the samples to ice between each sonication event. Sonicated samples were centrifuged for 30 min at 150,000g, and PGs released from the thylakoid floated to the surface of the solution. PGs were removed and combined with medium R with 0.7 mM Suc to achieve a Suc concentration of 0.5 M, which was then overlaid with medium R with 0.2 mM Suc and medium R with no Suc. The gradient was centrifuged for 90 min at 150,000g. The resulting floating band of PGs was removed, flash frozen in liquid N₂, and stored at −80°C.

**Antiserum Generation**

Nucleotide sequences encoding the soluble part of the M48 protein (amino acids 72–325) and the C termini of ABC1K3 (556–711) and ABC1K1 (578–582) were amplified by PCR. The resulting DNA fragments were ligated into restriction sites of the pET21a expression vector, coding for a C-terminal His affinity tag. The vector was transformed into BL21 Escherichia coli cells, and overexpressed protein was harvested from liquid culture after incubation in 1 mM isopropylthio-β-galactoside for 3 h at 37°C. Proteins were solubilized in 200 mM NaCl, 50 mM Tris, and 8 mM urea at pH 8 and purified on a nickel-nitrotriacetic acid agarose resin matrix, and polyclonal antibodies were raised in rabbits by injecting purified antigen.

**Immunoblotting**

Protein concentrations were estimated by the bicinchoninic acid (BCA) method (Smith et al., 1985) using a BCA kit (Pierce). Protein samples were solubilized in 1× Laemmli buffer (125 mM Tris-HCl, pH 6.8, 2% SDS, 5% β-mercaptoethanol, and 10% glycerol), heated for 10 min at 75°C, and separated on an SDS-PAGE gel (6% acrylamide stacking, 12% separation). Proteins were blotted to nitrocellulose, probed with purified anti-peptide M10, anti-ABC1K3, anti-ABC1K1, anti-A1, or anti-VTE1 serum (a gift of Dr. Dean DellaPenna), and visualized by the horseradish peroxidase-based enhanced chemiluminescence system. Densitometric analysis of relevant spots was performed using the ImageJ software program (http://rsweb.nih.gov/ij/).

**TEM**

Leaf tissue from three individuals of each genotype at each time point was harvested 1 h after the beginning of the photoperiod. Leaf margins and midribs were excluded, and the remaining leaf tissue was divided into 1-× 2-mm sections with a fresh razor blade. Sections were fixed in 2% glutaraldehyde, 2% paraformaldehyde, 0.1% tannic acid, and 70 mM PIPES buffer, pH 6.8, for 2 h and then washed three times in 70 mM PIPES buffer, pH 6.8. Tissues were fixed in 1% osmium tetroxide (OsO₄) and 70 mM PIPES, pH 6.8, for 2 h and washed three times in 70 mM PIPES, pH 6.8. Tissues were then stained in 2% uranyl acetate for 1 h and washed twice in ultrapure water. Fixed and stained tissues were carried through an acetone series of increasing concentrations. Dehydrated tissue was then embedded with Spurr’s resin (Electron Microscopy Sciences) in increasing concentrations of resin in acetone, according to the manufacturer’s instructions. Fully embedded tissue was cured in resin blocks at 60°C overnight. Cured resin blocks were sectioned and imaged at Electron Microscopy Services.

**SEM**

Two to 3 μL of purified PG sample was spotted onto a silica wafer. A 3-μL drop of 2% OsO₄ in 70 mM potassium phosphate, pH 7.2, was added to the 3-μL drop of the PG sample on the silica wafer. The buffered OsO₄ was allowed to remain in contact with the PGs for 1 h at 4°C. After 1 h, the wafers were floated on a droplet of 70 mM potassium phosphate buffer at pH 7.2 for 10 min. This was done three times at 4°C. The wafers were then floated on drops of 2% glutaraldehyde in 70 mM potassium phosphate, pH 7.2, for 1 h at 4°C. After 1 h, the wafers were floated on drops of 70 mM potassium phosphate, pH 7.2, for 10 min at 4°C. The wafers were then floated on drops of distilled water for 10 min at 4°C. The wafers were dehydrated by floating on first 25%, then 50%, then 75%, and finally 100% ethanol for approximately 10 min each at 4°C. The wafers were then critical point dried in 6% ethanol (Bal Tec; Leica Microsystems), mounted on specimen supports, and sputter coated with gold/palladium (Denton Vacuum). The wafers were viewed at 3 kV in a Hitachi S4000 scanning electron microscope (Hitachi High Technologies).

**In-Solution and In-Gel Digestion of Isolated PGs**

For in-solution digestion, isolated PGs were precipitated in 10% TCA overnight at 4°C. Precipitated proteins were pelleted by centrifugation and washed once with 100% acetone and once with 80% acetone, 10% methanol, and 0.1% acetic acid by incubating at −20°C for 1.5 h each. Washed pellets were resuspended in dimethyl sulfoxide and quantified by the BCA method. For gel-based separation and in-gel digestion, PG samples were lyophilized and solubilized in a modified Laemmli solubilization buffer (125 mM Tris-HCl, pH 6.8, 6% SDS, 10% β-mercaptoethanol, and 20% glycerol). Samples were shaken...
Proteome Analysis of Total Leaf Extracts

Wild-type plants (Col-0) were grown on soil for 30 d under a short-day cycle (10 h/14 h of light/dark) at 120 μmol photons m\(^{-2}\) s\(^{-1}\). The complete leaf rosette was then harvested, and proteins were immediately quantitatively extracted in the presence of SDS (in triplicate) as described in detail by Friso et al. (2011). Alternatively, plants were transferred and grown on soil for 2.5 weeks under similar conditions as above, but transferred to 520 μmol photons m\(^{-2}\) s\(^{-1}\) conditions. In the morning of day 6, leaf tissue was harvested and extracted as above (in triplicate).

Proteome Analysis by NanoLC-LTQ-Orbitrap and Data Processing

Peptides prepared from in-gel digestion and in-solution digestion were analyzed by data-dependent MS/MS using online LC-LTQ-Orbitrap (Thermo Electron) with dynamic exclusion, as described (Zybaivol et al., 2008). Peak lists (mgf format) were generated using DTA supercharge (version 1.19) software (http://msquant.sourceforge.net/) and searched with Mascot version 2.2 (Matrix Science) against a combined database containing the Arabidopsis genome with protein-coding gene models and 187 sequences for known contaminants (e.g. keratin and trypsin; a total of 33,013 entries) and concatenated with a decoy database where all the sequences were randomized; in total, this database contained 66,026 protein sequences. Offline calibration for all precursor ions was done as described by Oliinares et al. (2010). Each of the peak lists was searched using Mascot version 2.2 (maximum P = 0.01) for full tryptic peptides using a precursor ion tolerance set at ±6 ppm, fixed Cys carbamido methylation and variable Met oxidation, protein N-terminal acetylation, Asn/Gln deamidation, and maximally one missed cleavage allowed. The maximum fragment ion tolerance (MS/MS) was 0.8 D. For semitryptic peptides, the search was performed with a precursor ion tolerance set at ±3 ppm, fixed Cys carbamido methylation and variable Met oxidation, N-terminal acetylation, Gln deamidation, and maximally one missed cleavage allowed. The minimal ion score threshold was chosen such that a peptide false discovery rate below 1% was achieved. Using an in-house-written filter, the search results were further filtered as follows. For identification with two or more peptides, the minimum ion score threshold was set to 30. For protein identification based on a single peptide, the minimum ion score threshold was set to 33 and the mass accuracy of the precursor ion was required to be within ±3 ppm. The peptide false discovery rate was calculated as \(2 \times (\text{decoy hits}/\text{target + decoy hits})\) and was below 1%. The false discovery rate of proteins identified by two or more peptides was 2%. Peptides with less than seven amino acids were discarded. All mass spectral data (the mgf files reformatted as PRIDE XML files) are available via the Proteomics Informatics database (PRIDE) at http://www.ebi.ac.uk/pride/ with accession numbers 18969 to 18988.

Several Arabidopsis genes have more than one gene model, and in such cases the protein form with the highest number of matched spectra was selected; if two gene models had the same number of matched spectra, the model with the lower digit was selected. For quantification, each protein accession was scored for total spectral counts (SPC), unique SPC (uniquely matching to an accession), and adjusted SPC (Friso et al., 2011). The latter assigns shared peptides to accessions in proportion to their relative abundance using unique spectral counts for each accession as a basis. The NadSPC for each protein was calculated through division of adjSPC by the sum of all adjSPC values for the proteins from that gel lane. NadSPC provides a relative protein abundance measure by mass, whereas the normalized spectral abundance factor estimates relative protein concentration within a particular sample, as defined by Friso et al (2011).

Plastoglobular Functions

The PGCs of all pairwise combinations between PG (bait) genes and all single-gene probes of the Arabidopsis 22K Affymetrix microarray were calculated using three different software programs: the MetaOmGraph software program (http://metnetdb.org; Wurtele et al., 2007), the BAR expression anger (http://142.150.214.117/welcome.htm; Toutighi et al., 2005), and the ACT Web site (http://www.arabidopsis.leeds.ac.uk/act/index.php; Manfield et al., 2006). MetaOmGraph analysis used the publicly available Affyath1.data project containing normalized, averaged Arabidopsis experimental data sets obtained from NASCArrays (http://afymetrix.arabidopsis.info/) and PlexDB (http://plexdb.org) from 71 experiments and 424 microarray chips from diverse environmental and genotypic conditions and tissue types and developmental stages. Correlations were calculated using the Pearson correlation algorithm. Visualization of the MetaOmGraph-derived network was performed in Cytoscape version 2.8.0 (http://cytoscape.org/; Shannon et al., 2003), applying the force-directed layout algorithm. Coexpression analysis using the BAR expression anger was performed for each PG gene by searching in the NASCArrays 392 data set available at the Web site. Analysis at the ACT Web site was performed for each PG gene by using the “Co-expression analysis over available array experiments” option.

Analysis of Transcript Accumulation during Natural Senescence

Wild-type Arabidopsis Col-0 was grown on soil. Leaf tissue was selected from five time points during the course of natural leaf senescence: 1 = leaf rosette from plants beginning to bolt; 2 = leaf rosette from plants beginning to flower; 3 = senescing leaf approximately 10% chlorotic; 4 = senescing leaf approximately 50% chlorotic; 5 = senescing leaf approximately 50% chlorotic, 6 = leaf rosette in senescence. Total RNA was extracted using the RNeasy plant miniprep kit (Qiagen) according to the manufacturer’s instructions. Seven hundred nanograms of total RNA was used for the synthesis of cDNA using oligo(dT)\(_2\)\(_5\) primer and the SuperScript III cDNA synthesis kit (Invitrogen) according to the manufacturer’s instructions. cDNA samples were diluted to equal concentration by normalizing according to amplification of the ACTIN2 gene using 20 cycles. Each gene was then amplified for 25 cycles using an equal volume of template and an appropriate gene-specific primer pair. Signal intensity was quantified using the alpha Imager 2200 version 5.5 software package. The forward and reverse primers are as follows: for PAO/ACD1, 5'-GATGCCGAACATCATTGTCG-3' and 5'-CATCAGAAAGGGACACCCGAC-3'; for PPH, 5'-CAATCATTGTCGTCCTGGT-3' and 5'-CTACCATCATTGTCGACCTCC-3'; for DGC3, 5'-GCGGAGGACCATCTTTGTTACT-3' and 5'-GCGGAGGACCATCTTTGTTACT-3'; for ARB127, 5'-ATCCACCACGAAACCCCT-3' and 5'-ACAGATCCTGCGATAAGGAGG-3'; for MRS, 5'-GAAATCACCGCTTGAGTCAAGC-3' and 5'-GGTGTGTGGCGTCATGAT-3'; for ESTERASE, 5'-GTAACCTGTGTTACCTCC-3' and 5'-AAAATCCCGATGTCTGCGCC-3'; for ABC193, 5'-GCAAGCTGGTCGTTAACCC TC-3' and 5'-CAATCATTGCGATAAGGAGG-3'; for ABC194, 5'-TTCTCCGAGCTCACT-3' and 5'-ATGGTGTGGCGTCATGAT-3'; and for ACTIN2, 5'-GACACGGGCGCTCAGCAAGAATC-3' and 5'-GCAATCGCAGATGATGAGGAGAAGGA-3'.

Calculation of Protein Physicochemical Parameters

Parameters were calculated by the ProtParam tool (Gasteiger et al., 2005) available through the ExPasy Web site (http://expasy.org/tools/).

Sequence data from this article can be found in the GenBank/EMBL data libraries under the following accession numbers: AT3G49070 (FBN1a), AT3G22340 (FBN4), AT4G22240 (FBN1b), AT2G35490 (FBN2), AT2G35520 (ABC1K9), AT1G79600 (ABC1), AT3G13090 (ABC1K3), AT1G89110 (FBN7a), AT1G192170 (CCD4), AT4G32770 (VTE1), AT5G8740 (NDC1), AT1G35470 (DGAT3), AT2G41230 (FBN7b), AT1G32220 (FR-like), AT3G13200 (Unknown-1), AT2G49610 (FBN8), AT3G10130 (SOUL-like), AT2G41040 (Ub1-like), AT1G71180 (ABC1K5), AT1G66980 (AKR-like), AT2G34460 (FR-like), AT1G78140 (Ub1-like), AT3G26840 (DGAT3), AT3G24190 (ABC1K6), AT4G39730 (PLAT/LH2-1), AT3G34540 (Unknown-2), AT2G22270 (PLAT/LH2-2), AT3G07700 (ABC1K7), AT1G73750 (Unknown SAG), AT3G27110 (M48 metalloprotease), and AT5G41210 (Esterase1).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Five-day light-shifted wild-type Arabidopsis plant, representative of those used in this work.
Supplemental Figure S2. Scanning electron micrographs of PG preparations.

Supplemental Figure S3. Coexpression within gene sets of chlorophyllide biosynthesis and the ClpPR protease complex.

Supplemental Figure S4. PG genes preferentially maintain coexpression with other PG genes at higher FCCs.

Supplemental Figure S5. Coexpression relationships between PG genes and isoprenoid metabolism genes found in MetaOmGraph projected onto the isoprenoid pathway.

Supplemental Table S1. Experimental data of in-gel and in-solution PG proteome analysis.

Supplemental Table S2. Comparison of protein abundances in PG, total leaf, thylakoid, and stroma.

Supplemental Table S3. MetaOmGraph coexpression results: top 20.

Supplemental Table S4. Functional group enrichment of PG coexpressers using different software programs.

Supplemental Text S1. Testing and benchmarking of coexpression analysis tools.

ACKNOWLEDGMENTS

We thank Carole Daugherty (Cornell Center for Material Research) for critical assistance with the SEM analysis of PG preparations, Richard Medville of Electron Microscopy Sciences for the collection of TEM micrographs, and Dr. Dean DellaPenna (Michigan State University) for the generous donation of anti-VTE1 serum.

Received December 30, 2011; accepted January 19, 2012; published January 24, 2012.

LITERATURE CITED

Alboresi A, Dall’osto L, Aprile A, Carillo P, Roncaglia E, Cattivelli L, Bassi R (2011) Reactive oxygen species and transcript analysis upon excess light treatment in wild-type Arabidopsis thaliana vs a photosensitive mutant lacking zeaxanthin and lutein. BMC Plant Biol 11: 62

Aubert Y, Vile D, Pervent M, Aldon D, Ranty B, Simonneau T, Vavasseur A, Galaud J-P (2010) RD20, a stress-inducible caleosin, participates in sensitive mutant lacking zeaxanthin and lutein. BMC Plant Biol 11: 62

Austin JR II, Frost E, Vidi PA, Kessler F, Staehelin LA Beisel KG, Jahnke S, Hofmann D, Köppchen S, Schurr U, Matsubara S, Bailey JL, Whybom AG (2011) Reactive oxygen species and transcript analysis upon stress in Arabidopsis thaliana. Plant Cell 23: 430–443

Bassi R (2011) Chlorophyll breakdown in higher plants. Biochim Biophys Acta 1807: 977–988

Hu Q, Noll RJ, Li H, Makarov A, Hardman M, Graham Cooks R (2009) The Orbitrap: a new mass spectrometer. J Mass Spectrom 40: 430–443

Klimmek E, Sjödin A, Noutsos C, Leister D, Jansson S (2006) A defect in coenzyme Q biosynthesis is responsible for the respiratory deficiency in Saccharomyces cerevisiae abcl mutants. J Biol Chem 271: 18161–18168

Lundquist et al.

Cartieux F, Thibaud M-C, Zimmerli L, Lessard P, Sarrobert C, David P, Gerbault A, Robaglia C, Somerville S, Nussleume L (2003) Transcriptome analysis of Arabidopsis colonized by a plant-growth promoting rhizobacterium reveals a general effect on disease resistance. Plant J 36: 177–188

DalCorso G, Pesaresi P, Masiero S, Aseeva E, Schümann D, Finazzi G, Joliot P, Barbato R, Leister D (2008) A complex containing PGR1 and PCR5 is involved in the switch between linear and cyclic electron flow in Arabidopsis. Cell 132: 273–285

Do TQ, Hsu AY, Jonassen T, Lee PT, Clarke CF (2001) A defect in coenzyme Q biosynthesis is responsible for the respiratory deficiency in Saccharomyces cerevisiae abcl mutants. J Biol Chem 276: 18161–18168

Domingo B, Aebersold R (2010) Options and considerations when selecting a quantitative proteomics strategy. Nat Biotechnol 28: 710–721

Ducrues LJM, Morris WL, Hedley PE, Shepherd T, Davies HV, Millam S, Taylor MA (2005) Metabolic engineering of high carotenoid potato tubers containing enhanced levels of beta-carotene and lutein. J Exp Bot 56: 81–89

Eugeni Piller L, Besagni C, Ksas B, Rumeau D, Bréhélin C, Glauser S, Kessler F, Havaux M (2011) Chloroplastic lipid droplet type II NAD(P)H quinone oxidoreductase is essential for prenylquinone metabolism and vitamin K1 accumulation. Proc Natl Acad Sci USA 108: 14354–14359

Fitter DW, Martin DJ, Copley MJ, Scotland BW, Langdale JA (2002) GLK gene pairs regulate chloroplast development in diverse plant species. Plant J 31: 713–727

Friso G, Majeran W, Huang M, Sun Q, van Wijk KJ (2010) Reconstruction of metabolic pathways, protein expression, and homeostasis machineries across maize bundle sheath and mesophyll chloroplasts: large-scale quantitative proteomics using the first maize genome assembly. Plant Physiol 152: 1219–1230

Friso G, Olianas PD, van Wijk KJ (2011) The workflow for quantitative proteome analysis of chloroplast development and differentiation, chloroplast mutants, and protein interactions by spectral counting. Methods Mol Biol 775: 265–282

Fu F-F, Xue H-W (2010) Coexpression analysis identifies Rice Starch Regulator1, a rice AP2/EREBP family transcription factor, as a novel rice starch biosynthesis regulator. Plant Physiol 154: 927–938

Furt F, Oostende C, Wöhlharm J, Dale MA, Wertz J, Basset GJ (2010) A bimodal oxidoreductase mediates the specific reduction of phylloquinone (vitamin K) in chloroplasts. Plant J 64: 38–46

Gasteiger E, Hoogland C, Gattiker A, Duvaud S, Wilkins MR, Appel RD, Bairoch A (2005) Protein identification and analysis tools on the ExPasy server. In: WM Keller, ed. The Proteomics Protocols Handbook. Humana Press, Totowa, NJ, pp 571–607

Gaude N, Bréhélin C, Tischendorf G, Kessler F, Dörmann P (2007) Nitrogen deficiency in Arabidopsis affects galactolipid composition and gene expression and results in accumulation of fatty acid phenyl esters. Plant J 49: 729–739

Greenwood AD, Leech RM, Williams JP (1963) The osmiophilic globules of chloroplasts. I. Osmiophilic globules as a normal component of plastids of higher plants. Biochim Biophys Acta 78: 163–174

Havaux M, Emyere F, Porfiriova S, Rey P, Dörmann P (2005) Vitamin E protects against photoinhibition and photooxidative stress in Arabidopsis thaliana. Plant Cell 17: 3451–3469

Hörfenstein S, Krüthler B (2011) Chlorophyll breakdown in higher plants. Biochim Biophys Acta 1807: 977–988

Hu Q, Noll RJ, Li H, Makarov A, Hardman M, Graham Cooks R (2009) The Orbitrap: a new mass spectrometer. J Mass Spectrom 40: 430–443

 Huang F-C, Molnár P, Schwab W (2009) Cloning and functional characterization of carotenoid cleavage dioxygenase 4 genes. J Exp Bot 60: 3011–3022

Jasinski M, Sudre D, Schansker G, Schellenberg M, Constant S, Martinova E, Bovet I (2008) AtOSA1, a member of the Abc1-like family, as a new factor in cadmium and oxidative stress response. Plant Physiol 147: 719–731

Klimmek E, Sjödin A, Noutsos C, Leister D, Jansson S (2006) Abundantly and rarely expressed Lhc protein genes exhibit distinct regulation patterns in plants. Plant Physiol 140: 793–804

Lin W-D, Liao Y-Y, Yang TJW, Pan C-Y, Buckhout TJ, Schmidt W (2011) Coexpression-based clustering of Arabidopsis root genes predicts functional modules in early phosphate deficiency signaling. Plant Physiol 155: 1383–1402

Liu H, Sadygov RG, Yates JR III (2004) A model for random sampling and estimation of relative protein abundance in shotgun proteomics. Anal Chem 76: 4193–4201
Livingston AK, Cruz JA, Kohzuma K, Dhiangra A, Kramer DM (2010a) An Arabidopsis mutant with high cyclic electron flow around photosystem I (heel) involving the NADPH dehydrogenase complex. Plant Cell 22: 221–233

Livingston AK, Kanazawa A, Cruz JA, Kramer DM (2010b) Regulation of cyclic electron flow in C₃ plants: differential effects of limiting photosynthesis at ribulose-1,5-bisphosphate carboxylase/oxygenase and glycerolaldehyde-3-phosphate dehydrogenase. Plant Cell Environment 33: 1779–1788

Lohmann A, Schöttler MA, Brélécin C, Kessler F, Bock R, Calhoon EB, Dörmann P (2006) Deficiency in phytoquinone (vitamin K₁) methylation affects prenyl quinone distribution, photosystem I abundance, and anthocyanin accumulation in the Arabidopsis AtmenG mutant. J Biol Chem 281: 40461–40472

Majeran W, Friso G, Ponnala L, Connolly B, Huang M, Reidel E, Zhang C, Akasaka Y, Bluian NH, Sun Q, et al (2010) Structural and metabolic transitions of C4 leaf development and differentiation defined by microscopy and quantitative proteomics in maize. Plant Cell 22: 3309–3342

Manfield IW, Jen C-H, Pinney JW, Michalopoulos I, Bradford JR, Gilmartin PM, Westhead DR (2006) Arabidopsis Co-expression Tool (ACT): Web server tools for microarray-based gene expression analysis. Nucleic Acids Res 34: W504–W509

Mann M, Kelleher NL (2008) Precision proteomics: the case for high resolution and high mass accuracy. Proc Natl Acad Sci USA 105: 18132–18138

Mutwil M, Obro J, Willatts WG, Pessonn S (2008) GeneCAT: novel Webtools that combine BLAST and co-expression analyses. Nucleic Acids Res 36: W320–W326

Old WM, Meyer-Arendt K, Aveline-Wolf L, Pierce KG, Mendoza A, Peng L, Shikanai T (2006) Arabidopsis Co-expression Tool (ACT): Web server tools for microarray-based gene expression analysis. Nucleic Acids Res 34: W504–W509

Ozaki S, Ogata Y, Suda K, Kurabayashi A, Suzuki T, Yamamoto N, Iijima Y, Tsugane T, Fujii T, Konishi C, et al (2005) Coexpression analysis of tomato genes and experimental verification of coordinated expression of genes found in a functionally enriched coexpression module. DNA Res 12: 105–116

Peng L, Shikani T (2011) Supercomplex formation with photosystem I is required for the stabilization of the chloroplast NADH dehydrogenase-like complex in Arabidopsis. Plant Physiol 155: 1629–1639

Poon WW, Davis DE, Ha HT, Jonassen T, Rather PN, Clarke CF (2002) Isolation of an Arabidopsis mutant lacking vitamin E and identification of a vitamin E pathway gene5-1 mutant reveals a critical role for tocopherol cyclase essential for all tocopherol biosynthesis. Proc Natl Acad Sci USA 99: 12495–12500

Puthiyaveetil S, Kavanagh TA, Cain P, Sullivan JA, Newell CA, Gray JC, Robinson C, van der Giezen M, Rogers MB, Allen JF (2008) The ancestral symbiont sensor kinase CSK links photosynthesis with gene expression in chlorellas. Proc Natl Acad Sci USA 105: 10061–10066

Reinhold C, Niczypruk S, Beran KC, Jahns P (2008) Short-term down-regulation of zeaxanthin epoxidation in Arabidopsis thaliana in response to photo-oxidative stress conditions. Biochim Biophys Acta 1777: 462–469

Rey F, Gillet B, Rümmer S, Eymery F, Massimino J, Pellet G, Kuntz M (2009) Over-expression of a pepper plastid lipid-associated protein in tobacco leads to changes in plastid ultrastructure and plant development upon stress. Plant J 64: 483–494

Rochaix JD (2011) Regulation of photosynthetic electron transport. Biochim Biophys Acta 1807: 375–383

Rohde A, Morrell K, Ralph J, Goeminne G, Hostyn V, De Rycke R, Kushnir S, Van Doorselaere J, Josseleur J-P, Vuylsteke M, et al (2004) Molecular phenotyping of the pal1 and pal2 mutants of Arabidopsis thaliana reveals far-reaching consequences on phenylpropanoid, amino acid, and carbohydrate metabolism. Plant Cell 16: 2749–2771

Sandhu C, Hewel JA, Badis G, Talukder S, Liu J, Hughes TR, Emili A (2008) Evaluation of data-dependent versus targeted shotgun proteomic approaches for monitoring transcription factor expression in breast cancer. J Proteome Res 7: 1329–1341

Sawada Y, Toyooka K, Kuswhara A, Sakata A, Nagano M, Saito K, Hirai MY (2009) Arabidopsis bide acid:sodium symporter family protein S is involved in methionine-derived glucosinolate biosynthesis. Plant Cell Physiol 50: 1579–1586

Sawchuk MG, Donner TJ, Head F, Scarpella E (2008) Unique and overlapping expression patterns among members of photosynthesis-associated nuclear gene families in Arabidopsis. Plant Physiol 148: 1908–1924

Shannon P, Markiel A, Ozier O, Baliga NS, Wang JT, Ramage D, Amin N, Schwikowski B, Ideker T (2003) Cytoscape: a software environment for integrated models of molecular interaction networks. Genome Res 13: 2498–2504

Simkin AJ, Gaffé J, Alcaraz JP, Carde JR, Bramley PM, Fraser PD, Kuntz M (2007) Fibrillin influence on plastid ultrastructure and pigment content in tomato fruit. Phy tochemistry 68: 1545–1556

Simon-Plas F, Perraki A, Bayer E, Gerbeau-Pissot P, Mongrand S (2011) An update on plant membrane rafts. Curr Opin Plant Biol 14: 642–649

Singh DK, Maximova SN, Jensen PJ, Lehman BL, Ngugi HK, McNellis TW (2010) FIBRILLIN4 is required for plastoglobule development and stress resistance in Arabidopsis. Plant Physiol 156: 1281–1293

Singh DK, McNellis TW (2011) Fibrillin protein function: the tip of the iceberg? Trends Plant Sci 16: 432–441

Smith PK, Krohn RJ, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, Fujimoto EK, Goekoe NM, Olson BJ, Klenk DC (1985) Measurement of protein using bicinchoninic acid. Anal Biochem 150: 76–85

Steinhauser D, Usadel B, Luedemann A, Thimm O, Kopka J (2004) CSB: DB: a comprehensive systems-biology database. Bioinformatics 20: 3647–3651

Szymbanska R, Kruk J (2008) Plantochromanol, a “new” lipophilic antioxidant is synthesized by tocopherol cyclase in Arabidopsis leaves: the effect of high-light stress on the level of prenyl glycolipid antioxidants. In: IF Allen, E Gannt, JH Golbeck, B Osmond, eds, Photosynthesis: Beyond the Sunlight, pp 1518–1584

Szymbanska R, Kruk J (2010) Plantochinol is the main prenylquinol synthesized during acclimation to high light conditions in Arabidopsis and is converted to plantochromanol by tocopherol cyclase. Plant Cell Physiology 51: 537–545

Takabayashi A, Ishikawa N, Obassyhi T, Ishida S, Obokata J, Endo T, Sato F (2009) Three novel subunits of Arabidopsis chloroplastic NAD(P)H dehydrogenase identified by bioinformatics and reverse genetic approaches. Plant J 57: 207–219

Touche A, Krause-Buchholz U, Rödel G (2008) Ubiquinone biosynthesis in Saccharomyces cerevisiae: the molecular organization of O-methylase Coq3p depends on Abc1p/Coq8p. FEBS J 275: 1263–1275

Treviño M, Steinmuller M (1985) Composition and formation of plastoglobuli. II. Lipid-composition of leaves and plastoglobuli during bee leaf senescence. Planta 163: 91–96

Toufighi K, Brady SM, Austin R, Ly E, Provart NJ (2005) The Botany Array Resource: e-northerns, expression angling, and promoter analyses. Plant J 43: 153–163

Usadel B, Obassyhi T, Mutwil M, Giorgi FM, Bassel GW, Tranimoto M, Chow A, Steinhauer D, Persson S, Provart NJ (2009) Co-expression tools for plant biology: opportunities for hypothesis generation and caveats. Plant Cell Environment 32: 1633–1651

Valentin HE, Lincoln K, Moshiri F, Jensen PK, Qi Y, Venkatash TV, Karunananad B, Basmis SR, Norris SR, Savidge B, et al (2006) The Arabidopsis vitamin E pathway gene5-1 mutant reveals a critical role for vitamin E in seed development and stress resistance in apple and Arabidopsis. Plant Physiol 148: 1281–1293

Vidi PA, Kessler F, Brélécin C (2006) Tocopherol cyclase (VTE1) localization and vitamin E accumulation in chloroplast plastoglobule lipoprotein particles. J Biol Chem 281: 11225–11234

Vidi PA, Kessler F, Brélécin C (2007) Plastoglobules: a new address for targeting recombinant proteins in the chloroplast. BMC Biotechnol 7: 4

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Wurtele E, Li L, Berleant D, Cook D, Dickerson J, Ding J, Hofmann H, Lawrence M, Lee E-k, Li J, et al (2007) Concepts in plant metabolomics. In BJ Nikolau, ES Wurtele, eds, Systems Biology Tools for Arabidopsis. Concepts in Plant Metabolomics. Springer, Dordrecht, The Netherlands, pp 145–157

Xie LX, Hsieh EJ, Watanabe S, Allan CM, Chen JY, Tran UC, Clarke CF (2011) Expression of the human atypical kinase ADCK3 rescues coenzyme Q biosynthesis and phosphorylation of Coq polypeptides in yeast coq8 mutants. Biochim Biophys Acta 1811: 348–360

Xu CC, Jeon YA, Hwang HJ, Lee C-H (1999) Suppression of zeaxanthin epoxidation by chloroplast phosphatase inhibitors in rice leaves. Plant Sci 146: 27–34

Ytterberg AJ, Pelletier JB, van Wijk KJ (2006) Protein profiling of plastoglobules in chloroplasts and chromatoplasts: a surprising site for differential accumulation of metabolic enzymes. Plant Physiol 140: 984–997

Zbierzak AM, Kanwischer M, Wille C, Vidi PA, Giavalisco P, Lohmann A, Briesen I, Porfirova S, Bréhélin C, Kessler F, et al (2010) Intersection of the tocopherol and plastoquinol metabolic pathways at the plastoglobule. Biochem J 425: 389–399

Zhang R, Wise RR, Struck KR, Sharkey TD (2010) Moderate heat stress of Arabidopsis thaliana leaves causes chloroplast swelling and plastoglobule formation. Photosynth Res 105: 123–134

Zybailov B, Coleman MK, Florens I, Washburn MP (2005) Correlation of relative abundance ratios derived from peptide ion chromatograms and spectrum counting for quantitative proteomic analysis using stable isotope labeling. Anal Chem 77: 6218–6224

Zybailov B, Friso G, Kim J, Rudella A, Rodriguez VR, Asakura Y, Sun Q, van Wijk KJ (2009) Large scale comparative proteomics of a chloroplast Clp protease mutant reveals folding stress, altered protein homeostasis, and feedback regulation of metabolism. Mol Cell Proteomics 8: 1789–1810

Zybailov B, Rutschow H, Friso G, Rudella A, Emanuelsson O, Sun Q, van Wijk KJ (2008) Sorting signals, N-terminal modifications and abundance of the chloroplast proteome. PLoS ONE 3: e1994