The Chloroplast Envelope Proteome and Lipidome

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1 The Chloroplast envelope proteome and lipidome

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Abstract The lipid and protein components of the two envelope membranes, which delimit the chloroplast from the surrounding cytosol, have been extensively analyzed. Envelope membranes contain a wide diversity of glycolipids, pigments and prenylquinones and play a key role in their synthesis, and also in the formation of various lipid-derived signaling molecules (chlorophyll precursors, abscisic acid and jasmonate precursors, for instance). Many of the enzymes involved were identified by proteomics. Here, we present a curated protein list established from chloroplast envelope proteomes analyzed by different groups. The envelope proteome contains key proteins involved in the regulation of metabolic pathways, in cell signaling (and especially in plastid-to-nucleus signaling), in stress responses, etc. A series of transport systems for proteins, metabolites and ions have also been identified by proteomics. Chloroplasts have had a long and complex evolutionary past and integration of the envelope membranes in cellular functions is the result of this evolution. The lipid and protein equipments of this plastid-specific membrane system reflect both its prokaryotic and eukaryotic origin. (167 words)

Key words Galactolipids, Carotenoids, Prenylquinones, Chlorophyll, Proteomics, Membrane proteins

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1 Introduction

Chloroplasts present three major structural regions: (a) a highly organized internal membrane network formed of flat compressed vesicles, the thylakoids, (b) an amorphous background rich in soluble proteins and ribosomes, the stroma and (c) a pair of outer membranes, the chloroplast envelope. The two limiting envelope membranes are actually the only permanent membrane structure of the different types of plastids (proplastids, chloroplasts, chromoplasts, etioplasts…); they are present in every plant cell, with very few exceptions (such as the highly specialized male sexual cells). Plastids are semi-autonomous organelles, with a wide structural and functional diversity, and unique biochemical pathways. They are able to transcribe and translate the information present in their own genome but are strongly dependent on imported proteins that are encoded in the nuclear genome and translated in the cytoplasm. The chloroplast genome encodes about 80 to 100 proteins, while between 2500 and 3500 nuclear-encoded proteins are predicted to be targeted to the chloroplast. The envelope membranes are essential to this process since (a) the tight co-ordination between the expression of plastidial and nuclear genomes requires plastid-to-nucleus signaling and (b) proteins transport across the envelope involves a complex transmembrane machinery.

Chloroplasts are crucial for plant cell metabolism. Performing photosynthesis, they are the site of carbon dioxide reduction and its assimilation into carbohydrates, amino acids, fatty acids, and terpenoid compounds. They are also the site of

nitrite and sulfate reduction and their assimilation into amino acids. The envelope membranes, as the interface between plastids and the surrounding cytosol, control the uptake of raw material for all synthesis occurring in the plastids and regulate the export to the cytosol of the newly synthesized molecules. Envelope membranes are therefore essential for the integration of plastid metabolism within the cell.

The participation of chloroplast envelope membranes in most of these particular aspects of chloroplast integration within the plant cell has been analyzed in detail in various reviews to which the reader is referred for more specific information (see Douce and Joyard, 1979; 1990; Bölter and Soll 2001; Jarvis and Soll 2002; Hiltbrunner et al. 2001; Aldridge et al. 2005; Weber et al. 2005; Nott et al. 2006; Maple et al. 2005; Maple and Møller 2006; Dörmann 2007; Jouhet et al. 2007; Block et al. 2007 and references herein). The reader is also referred to different chapters in this volume dealing with envelope membranes and their role in chloroplast interaction with the environment.

The aim of this review is to focus on our understanding of the lipid and protein composition of chloroplast envelope membranes that were analyzed in detail owing to the purification of chloroplast envelope membranes from spinach or *Arabidopsis*. In particular, we want to link the large body information recently obtained by a series of proteomic studies to our current knowledge of chloroplast envelope functions, and therefore to provide a more clear picture of the role of this membrane system within the plant cell.

2 Chloroplast envelope lipidome

Envelope membranes are very lipid-rich (compared to thylakoids, mitochondrial membranes or endomembranes) and this confers to these membranes a low density. In an extensive envelope lipid analysis (performed from 122 mg of dried lipids extracted from purified spinach chloroplast envelope membranes), Siebertz et al. (1979) determined that lipids accounted for 69% of the membrane dry weight. Further analysis of both envelope membranes demonstrated that the outer envelope membranes were mostly responsible for such a high lipid to protein ratio: they contain about 2.5 to 3 mg lipids/mg proteins, resulting in a very low density (1.08 g/cm^3, Block et al. 1983). The low protein content in the outer envelope may also result in large protein-free lipid bilayer areas that could be responsible for the patchy distribution of some envelope proteins when analyzed by confocal microscopy. The lipid to protein ratio of the inner membrane is around 1 mg lipids/mg proteins, corresponding to a density of 1.13 g/cm^3, Block et al. 1983).

2.1 Glycerolipids

Like other plastid membranes, envelope membranes are made of polar neutral lipids containing galactose and called galactolipids (Benson 1964). The galactose

residue in monogalactosyldiacylglycerol (MGDG) is bound to the *sn*-3 position of the glycerol backbone with a β-anomeric linkage (βGalD), whereas the head group of digalactosyldiacylglycerol (DGDG) is characterized by a terminal α-galactose moiety (1 → 6) linked to the inner β-galactose residue (αGal(1 → 6)βGalD). Another glycolipid, the sulfolipid SQDG (1,2-di-*O*-acyl-3-*O*-(6'-deoxy-6'-sulfo-α-D-glucopyranosyl)-*sn*-glycerol; sulfoquinovosyldiacylglycerol) is also characteristic of plastid membranes. In addition to the main MGDG (1,2-di-*O*-acyl-3-*O*-β–D-galactopyranosyl-*sn*-glycerol) and DGDG (1,2-di-*O*-acyl-3-*O*-(6'-*O*-α-galactopyranosyl-β-D-galactopyranosyl)-*sn*-glycerol), a series of higher homologues formed by sequential addition of α-D-galactopyranosyl residues to C6 of the terminal galactose of the preceding homologue, have been identified in chloroplast subfractions (see below and Table 1). It is now clear that the higher homologues trigalactosyldiacylglycerol (TGDG) and tetragalactosyldiacylglycerol (TeGDG are due to the functioning of a galactolipid:galactolipid galactosyltransferase (GGGT) during the course of envelope purification. The same is true for acylated DGDG which represents a significant proportion of glycerolipids in envelope preparations (Table 1): it is formed at low pH in leaf homogenates after destruction of cellular compartmentation (Siebertz et al. 1979).

Roughly 20-25% of envelope acyl lipids are phospholipids, mostly phosphatidylcholine (PC), and phosphatidylglycerol (PG) representing 5 to 15% of the total glycerolipids in these membranes (Tables 1-3). Again, these phospholipids are not distributed evenly in chloroplast membranes. The outer envelope membrane contains significantly more phospholipids than the other chloroplast membranes, mostly PC, which represents 30-35% of the outer envelope membrane lipids. Using intact chloroplasts, the outer surface of the outer envelope membrane can be probed directly with antibodies, proteases or lipases. For instance, Billecocq et al. (1972) and Billecocq (1975) have shown, by means of specific antibodies, that galactolipids and sulfolipid are present in the cytosolic leaflet of the outer envelope membrane. By using phospholipase C treatment of isolated intact chloroplasts, Dorne et al. (1985, 1990) have demonstrated that the envelope PC is concentrated in the outer leaflet of the outer envelope membrane and absent from the inner envelope membrane and the thylakoids (see also Table 2). In some preparations, very low amounts of PE (phosphatidylethanolamine) can be found. This likely reflects a contamination by extraplastidial membranes such as plant mitochondria or peroxisomes, that contain only phospholipids: mostly PC and PE.

Analyses of whole envelope membranes (containing both outer and inner envelope membranes) from spinach chloroplasts, cauliflower proplastids or pea etioplasts led to the conclusion that the glycerolipid pattern of envelope membranes from all plastid types is almost identical.

In plants grown under normal conditions, galactolipids are indeed restricted to plastid membranes. Determination of the glycerolipid composition of isolated plant membranes is therefore a good way to probe their purity but this should be used cautiously. When plants are deprived of P_i, DGDG strongly and specifically increases (Härtel et al. 1998, 2000; Klaus et al. 2002), and is present not only in plastid membranes but also in several membranes disconnected from plastid membranes (see section 3 below).

2.2 Composition, positional distribution and pairing of fatty acids in envelope lipids

In contrast to their conserved head group structures, the fatty acids of glycerolipids exhibit a high variability in chain lengths, degree of unsaturation and distribution to the *sn*-1 and *sn*-2 position of the glycerol backbone resulting in a high number of different molecular species originating from complex biosynthetic pathways. For instance, there are two main classes of galactolipids (Heinz 1977; Heinz and Roughan 1983) issued from two specific sources of DAG and notably represented at the level of MGDG. The prokaryotic-type class of galactolipids contains 16-carbon fatty acids at *sn*-2 position of glycerol. The eukaryotic-type class contains only 18-carbon fatty acids at *sn*-2 position of glycerol (Siebertz et al. 1979; Frentzen et al. 1983). Some plants such as *Arabidopsis* and spinach have both prokaryotic-type $MGDG_P$ and eukaryotic-type $MGDG_E$, whereas other plants such as pea or cucumber have only $MGDG_E$. DGDG is mostly of eukaryotic-type in all plants. PG contains exclusively prokaryotic DAG and is unique since it contains a $16:1_{trans}$ fatty acid at the *sn*-2 position of the glycerol backbone (Fritz et al. 2007). PC is a typical eukaryotic lipid.

Actually, the fatty acid distribution established for lipids of whole leaves was also found at the level of envelope membranes, as shown by Siebertz et al. (1979) who analyzed the fatty acid composition, positional distribution and pairing of envelope lipids from spinach (Table 1). In spinach, envelope lipids have a slightly increased level of 16:0 fatty acids as compared to thylakoids. The envelope MGDG contains high proportions of 16:3 and 18:3 fatty acids, thus two main species are found in MGDG: 16:3/18:3 (*sn*-1/*sn*-2) and 18:3/18:3, i.e. $MGDG_P$ and $MGDG_E$. This is also the case for DAG (diacylglycerol), TGDG and TeGDG, thus providing further evidence for these lipids to derive from MGDG during the course of envelope purification. In contrast, the envelope DGDG has the lowest level of 16:3 fatty acids, but the highest proportion of 16:0 fatty acids. Therefore, the main species in DGDG are 18:3/18:3, 18:3/16:0 and 16:0/18:3 with small amounts of 18:3/16:3 that could derive from the action of GGGT on MGDG. SQDG has roughly equal proportions of 16:0 and 18:3 fatty acids. Therefore, the major SQDG species identified in spinach envelopes are 16:0/16:0 and both 18:3/16:0 and 16:0/18:3. However, one should keep in mind that spinach contains a higher proportion of SQDG with a prokaryotic structure, whereas in plants that lacks procaryotic MGDG (such as wheat or cucumber), SQDG is exclusively eukaryotic (Bishop et al. 1985). Like thylakoid PG, the envelope PG contains $16:1_{trans}$ fatty acids. Two main species were identified in spinach envelopes with $18:3/16:1_{trans}$ or 18:3/16:0 combinations. Finally, the DAG backbone of PC displays many species of similar proportions (mostly 16:0/18:3 and 18:3/18:3 combinations). Interestingly, 16:0 fatty acids in the 16:0/18:3 species of PC are localized nearly exclusively at the *sn*-1 position of the glycerol backbone. In contrast, the percentage of eukaryotic DGDG (i.e. with 16:0 at the *sn-1* position) is highly plant-specific: 25% in spinach but almost 100% in *Vicia* (Rullkötter et al. 1975); and is also increased under phosphate deprivation (see below).

In summary of these analyses of spinach envelope lipids, we may say that specificities of DAG backbone known from lipid mixtures extracted from whole leaves are also found in envelope membranes. MGDG and DGDG have distinct DAG backbone (with 18:3/16:3 combination in MGDG and 18:3/16:0 in DGDG), whereas SQDG, PG and PC each displays a unique DAG pattern. This suggests differences in specificities in handling of acyl groups subsequent to the primary incorporation into DAG backbone of polar lipids.

2.3 Other lipid-soluble envelope constituents

Plant membranes, and especially plastid membranes, contain a wide diversity of compounds deriving from the isoprenoid biosynthetic pathway (Lange & Ghassemian 2003). Carotenoids (C40) and chlorophylls (which contain a C20 isoprenoid side-chain) are pigments essential for photosynthesis, whereas plastoquinone, phylloquinone and ubiquinone (all of which contain long isoprenoid side-chains) participate in electron transport chains. Many of them have been identified as constituents of chloroplast envelope membranes, which play a key role in their synthesis (Douce and Joyard 1990). Furthermore, chloroplasts contain biosynthetic pathways for phytohormones derived from isoprenoid intermediates, such as gibberellins (C20) and abscisic acid (C15), but despite some evidences (Helliwell et al. 2001), we are still missing a global view of the participation of envelope membranes to the production of signaling terpenoid derivatives.

2.3.1 Carotenoids

In contrast to thylakoids, envelope membranes from chloroplasts and non-green plastids are yellow, due to the presence of carotenoids and the absence of chlorophyll (Table 2). Carotenoids represent about 0.2% of the total envelope lipid weight (about 10 µg/mg protein) (Siebertz et al. 1979). Violaxanthin is the major carotenoid whereas thylakoids are richer in β-carotene (Jeffrey et al. 1974). Interestingly, whereas most carotenoids are in their stable *trans* configuration, we observed that 9'-cis-neoxanthin preferentially accumulates (compared to trans-neoxanthin) in chloroplast envelope membranes. In thylakoids, a transmembrane violaxanthin cycle is organized with de-epoxidation taking place on the lumen side and epoxidation on the stromal side of the membrane (Yamamoto et al. 1999). In the envelope, violaxanthin undergoes a light-induced decrease without a corresponding increase in zeaxanthin: the envelope lacks a violaxanthin cycle and the decrease of violaxanthin parallels the decrease in thylakoids (Siefermann-Harms et al. 1978). Therefore, envelope membranes prepared from leaves kept in the dark have up to 3.5 more violaxanthin than lutein + zeaxanthin, whereas in envelope membranes prepared from illuminated leaves, this ratio is much lower (0.75). An exchange of violaxanthin between the thylakoids and envelope but not of zeaxanthin was thus concluded to occur. Recently, Yamamoto (2006) observed

that the relative solubility of violaxanthin and zeaxanthin in MGDG, DGDG and phospholipids could explain the differential partitioning of violaxanthin between the envelope and the thylakoids and hypothesized that violaxanthin cycle thus links the thylakoids and the envelope for signal transduction of light stress.

2.3.2 Other pigments

Although devoid of chlorophyll, envelope membranes contain low amounts of chlorophyllide and protochlorophyllide (Pineau et al. 1986; 1993), thus suggesting that part of chlorophyll biosynthetic pathway is present in envelope membranes. Since the development of photosynthetic membranes is dependent upon the synthesis of chlorophylls and their specific integration into photosynthetic complexes in thylakoids, one can question why the envelope membranes contain chlorophyll precursors. Reinbothe et al. (1995, 2000) suggested that protochlorophyllide could regulate plastid import of pPORA, which would couple pPORA import to synthesis of its substrate. Furthermore, there is some evidence that the synthesis of chlorophyll precursors in envelope membranes is involved in intracellular signaling for the control of chloroplast development. The importance of chloroplast envelope membranes in these processes was confirmed by proteomics (see below).

2.3.3 Quinones

Like thylakoids, chloroplast envelope membranes contain several prenylquinones (Lichtenthaler et al. 1981; Soll et al. 1985): plastoquinone-9, phylloquinone K1, α-tocoquinone and the chromanol, α-tocopherol. However, the relative quinone composition of the envelope differs distinctively from that of thylakoid membranes. The outer envelope membrane contains more α-tocopherol than the inner one, although this prenylquinone is the major one in both membranes. On the contrary, plastoquinone-9, the major thylakoid prenylquinone, is present in higher amounts in the inner envelope membrane than in the outer one. Mutant characterization revealed that tocopherol protects plant lipids against oxidative stress (Havaux et al. 2005). The roles of tocopherol in plants are more complex than previously anticipated: further aspects such as interference with signaling pathways, subcellular/subplastidial localization and interactions with the chlorophyll degradation pathway have to be taken into consideration (reviewed by Dörmann 2007).

2.3.4 Sterols

Plastid membranes contain very few sterols (7 µg/mg protein) compared to extraplastidial membranes. Hartmann-Bouillon and Benveniste (1987) found that the major sterol in envelope membranes was stigmat-7-enol, whereas in the microsomes from the same tissue, it is α-spinasterol, thus suggesting that the presence

of sterols in envelope membranes is not caused by contamination by sterol-rich membranes (endoplasmic reticulum or plasma membrane). As mentioned above, Siebertz et al. (1979) identified only traces of sterylglycosides in more than 100 mg lipids from envelope membranes.

3 Functions of chloroplast envelope lipids

Glycerolipids synthesized in the envelope are necessary for thylakoids biogenesis (Kobayashi et al. 2007). Understanding the function of these lipids in plant cells is a key question that can be addressed by following the phenotypes of mutants impaired in genes encoding envelope lipid biosynthetic enzymes and/or by analyzing how plants adapt to fluctuating environmental conditions (see below).

MGD1 is the predominant MGD synthase in leaves (Jarvis et al. 2000; Awai et al. 2006; Kobayashi *et al*. 2007). In the deletion mutant of *mgd1*, invagination of the inner envelope was visible, suggesting a blockage in membrane trafficking from inner envelope to nascent thylakoids in absence of MGDG synthesis (Kobayashi et al. 2007). Moreover the deletion mutants of *mgd1* showed a complete impairment of photosynthetic growth with an arrest at embryo development (Kobayashi et al. 2007). DGDG synthases, DGD1 and DGD2, catalyze transfer of galactose from UDP-galactose to MGDG and are present in the outer envelope (Kelly and Dörmann 2002; Kelly *et al.* 2003). The *dgd1* mutant is strongly deficient in photosynthesis (Dörmann *et al.* 1995, Härtel *et al.* 1997) and protein import (Chen and Li 1998). Two proteins involved in PG synthesis, the PG-phosphate synthases PGP1 and PGP2 (Frentzen 2004; Muller and Frentzen 2001), were found in proteomic analyses of *Arabidopsis* chloroplast envelope membranes (Ferro *et al.* 2003; see below). The PGP1 protein is essential for chloroplast differentiation and for the biosynthesis of thylakoids PG (Babiychuk *et al.* 2003). SQDG was demonstrated to interact with an annexin in a Ca^{2+} dependent manner on the outer surface of chloroplast, suggesting a role of SQDG in the binding of this protein (Seigneurin-Berny *et al.* 2000). Along with PG, SQDG contributes to maintaining a negatively charged lipid-water interface, which is presumably required for proper function of photosynthetic membranes (Frentzen 2004).

Galactolipids synthesized in the envelope are also important for overall membrane lipid homeostasis of the plant cell. Under Pi starvation, a form of DGDG with a specific fatty acid signature, e.g. with 16:0 at *sn*-1 position of glycerol and 18:2 at *sn*-2 position, corresponding to a eukaryotic-type of DGDG, is particularly abundant in the cell (Härtel et al. 1998, 2000; Klaus et al. 2002). DGDG can thus form up to 20-25 % of the lipid content of mitochondria membranes (Jouhet et al. 2004), and plasma membrane (Andersson et al. 2003) and is also present in the tonoplast (Andersson et al. 2005). It was shown that mitochondrial DGDG is formed in the plastid envelope and then transported to mitochondria (Jouhet et al. 2004).

The particular concentration of PC in the outer leaflet of the outer envelope membrane suggests a role of PC in a connection with extra-plastidic compart-

ments. Studies with labeled lipid precursors indicated that PC synthesized in ER provides its DAG-backbone to chloroplast eukaryotic glycerolipids (Heinz and Harwood 1977; Slack et al. 1977). Although PC phospholipases are not present in chloroplasts, envelope PC can represent an intermediate step in the lipid transfer process.

The formation and trafficking of lipid synthesis intermediates in the chloroplast envelope may coordinate chloroplast development with overall cell development. DAG and PA are both key intermediates of the glycerolipid metabolism and both, and especially PA, are now recognized as major signaling lipids in plants (Testerink and Munnik 2005). Eukaryotic PA is not detected in the envelope in standard conditions. Only a very small pool of prokaryotic PA can be detected in chloroplasts (Fritz et al. 2007). Prokaryotic PA is the last common precursor shared by prokaryotic galactolipids and plastidial PG, which is exclusively prokaryotic. However a recent report indicated that artificial formation of eukaryotic PA in the envelope can induce conversion of eukaryotic PA into eukaryotic PG which severely reduced plant growth (Fritz et al. 2007). Several envelope proteins recently characterized, TGD1, TGD2 and TGD3, contribute to the transport of PA in the envelope membranes (Xu et al. 2005; Awai et al. 2006; Lu et al. 2007). Lipid metabolism is strongly affected in the three *tgd* mutants, with the largest effects being the reduction of the contents of eucaryotic MGDG and DGDG. It was proposed that TGD proteins can control the flux of eukaryotic PA between the two envelope membranes and, altogether, establish a link between lipid metabolism in ER and thylakoids development.

4 Chloroplast envelope proteome

4.1 From protein lists to a snapshot of the chloroplast envelope proteome

Proteomics, by combining the interest of targeted approaches (made possible by the preparation of envelope membranes from highly purified chloroplasts, especially from *Arabidopsis*) together with the availability of an increasing number of genome sequences (see for instance *The Arabidopsis Genome Initiative* 2000), proved to be a formidable tool to identify new proteins and therefore new functions residing in chloroplast envelope membranes. Before, we only had a very limited knowledge of the envelope protein equipment, despite many biochemical and physiological studies that characterized enzymatic and transport activities in purified envelope membranes and chloroplasts (Douce and Joyard, 1979, 1990). Table 4 presents our present knowledge of the chloroplast envelope proteome obtained by combining data from Ferro et al. (2002, 2003) and Froehlich et al. (2003). This "curated" list (see legend to Table 4) contains 226 proteins that we expect to be

envelope proteins. We suspect that proteins possibly residing in another cell compartment (stroma, thylakoids, mitochondria, nuclei, etc...) represent about 20% of the proteins identified by Ferro et al. (2002, 2003) and about 45% of those identified by Froehlich et al. (2003). This difference is mostly due to differences in sample preparation of envelope membrane fractions prior to proteomic analyses (see below). Key questions are raised by the comparison between the original (published) protein lists and the list in Table 4.

One generally considers that all membrane proteins should be hydrophobic, but the majority of the proteins identified by proteomics in membranes are not. Thus, where do we put the limit between a "true" envelope protein, a protein of the intermembrane space, an envelope-bound protein and a stromal protein, for instance? This is an open question and the answer is not simple. Another question is that of dual localization, although this is likely to concern only a minor proportion of the proteins.

Furthermore, it remains rather difficult to distinguish just by sequence analyses between envelope proteins that are likely residing within the membranes or are just envelope-bound. Proteins expected to be inner envelope membrane intrinsic proteins are metabolite and ionic transporters, components of the protein import machinery, and enzymes. Proteins of the last two categories as well as channel-forming proteins are expected to be located in the outer envelope membrane, most of them being devoid of any predictable transit sequence. Envelope preparations are expected to contain many "envelope-bound proteins": this is probably the case for some proteases and chaperones, for several putative RNA-binding proteins as well as enzymes like carbonic anhydrases and lipoxygenase. Indeed, many enzymes of carbon metabolism that are clearly identified as stroma enzymes are active in the vicinity of the inner face of the inner envelope membrane. It would not be too surprising that this would be the case for several proteins among the hundred identified by Froehlich et al. (2003) that we suspect to be stroma proteins.

4.2 Strategies for membrane proteomics reflect physico-chemical properties of envelope proteins

The main differences between published envelope proteomes reflect the strategies used (Figure 1). Froehlich et al. (2003) used *Arabidopsis* envelope preparation directly, without any fractionation, together with off-line multidimensional protein identification technology (off-line MUDPIT) to analyze the envelope proteome. In contrast, Ferro et al. (2002, 2003) analyzed envelope proteins from spinach and *Arabidopsis* chloroplasts by using a set of complementary methods (Seigneurin-Berny et al. 1999; Ferro et al. 2002, 2003; Ephritikhine et al. 2004) involving different extraction procedures and analytical techniques, i.e. solubilization in chloroform/methanol, and alkaline and saline treatments, prior to mass spectrometry analyses. Therefore, Ferro et al. (2002, 2003) chose to introduce a bias in their studies, since no "crude" envelope preparations were analyzed. The rationale behind this choice was to focus on the core of envelope membrane proteins, the

more peripheral proteins being excluded since it was difficult to choose –as mentioned above- between stromal (or to a lesser extent cytosolic) contamination and functional association to the envelope membrane. The envelope subfractions obtained were separated by 1D SDS-PAGE, followed by in-gel trypsin digestion and the tryptic fragments were analyzed by LC-MS/MS. For more details concerning this strategy, the reader is referred to several recent methodological reviews (Marmagne et al. 2006; Salvi et al. 2007a,b; Seigneurin-Berny et al. 2008).

4.2.1 Hydrophobic and soluble proteins in envelope membranes

The main interest of using different procedures to extract envelope proteins is to cover a wide range of protein dynamics and hydrophobicity. Ferro et al. (2003) identified more than 100 proteins in envelope membranes from *Arabidopsis* chloroplasts: about two third (69/112) were found from only one extraction method and not from any of the two other ones. Indeed chloroform/methanol extraction, alkaline and saline washings of *Arabidopsis* envelope membranes allowed the identification of 37, 51 and 74 proteins, respectively, by mass spectrometry (Ferro et al. 2003).

Chloroform/methanol is the most stringent method and alkaline treatment is more stringent than saline treatment with regard to the recovery of hydrophobic proteins. Organic solvent extraction appears to select for low Mr and high hydrophobicity with a low contamination with hydrophilic proteins (Ferro et al. 2002, 2003). This extraction method allowed the identification of proteins that are likely to be low abundance transporters. For instance, among the chloroform-methanol soluble proteins, Ferro et al. (2003) identified a new phosphate transporter representing only a minute fraction (about 0.1%) of the total envelope proteins. Although being less stringent with respect to hydrophobicity, alkaline and saline treatments allowed the identification of a few highly hydrophobic proteins altogether with more hydrophilic ones. Most of the proteins identified from NaCl treatment are predicted to contain no or only one transmembrane domain (Ephritikhine et al. 2004). NaOH treatment appears to be a good compromise to retrieve a wide range of proteins with different physico-chemical properties: this procedure clearly selects for more intrinsic proteins when compared to saline treatment since less hydrophilic contaminants are recovered (Ephritikhine et al. 2004). In addition, chloroform/methanol solubilize the most hydrophobic envelope proteins: almost all *Arabidopsis* envelope proteins identified in the chloroform/methanol extract have a Res/TM (Res stands for number of amino acid residues; TM for predicted transmembrane domains) ratio below 200 (31 among 37 identified proteins). As expected, more “soluble” proteins (Res/TM above 600) were identified in NaCl-washed envelope membranes, even when compared to NaOH-washed membranes (Ferro et al. 2003). In contrast, the envelope proteome identified by using unfractionated envelope preparations contains a large number of proteins (about 350) among which a very high proportion (70%) are devoid of any transmembrane domain (Froehlich et al. 2003). This value would be even much higher if the expected contaminant proteins (see table 4) were taken into ac-

count. As expected, the number of transmembrane domains is related to protein functions (Figure 2). Putative transporters represent the majority of envelope proteins with several α-helices constituting transmembrane domains: most of the 5% of envelope proteins having more than 10 transmembrane domains are putative transporters. Within this functional class, only a small proportion is devoid of transmembrane domains: these proteins have, in general, a β-barrel structure and were shown to reside to the outer envelope. In contrast, only a few of the numerous envelope proteins of the "Metabolism" class have transmembrane domains. This is also true for proteins of the "Chaperone & protease" class. However, one should keep in mind that not all "true" membrane protein have transmembrane domains, such as to porin-type proteins, monotopic proteins or proteins post-translationally modified by lipidic anchors. For instance, AtMGD1, the enzyme involved in the synthesis of MGDG, the major plastid membrane lipid, is a monotopic inner envelope membrane protein without any transmembrane α-helice (Miège et al. 1999).

4.2.2 Chloroplast envelope membranes are rich in basic proteins

Data in table 4 also demonstrates that envelope membranes contain numerous basic proteins. Whatever the extraction procedure, most of the proteins identified from plastid envelope fractions were shown to be basic (Ferro et al. 2003). A similar conclusion was made from *in silico* studies by Sun et al. (2004). Almost half of the envelope proteins likely to reside at the inner membrane have an isoelectric point higher than 9.0. When we consider only putative transporters, this value goes up to 70%. Thus, selection of basic proteins by chloroform/methanol extraction in chloroplast envelope membranes (Ferro et al. 2002, 2003) is due to the actual nature of the envelope proteins rather than to a specific bias of the extraction procedure. This was confirmed in a comparative survey of different plant membrane proteins extracted by chloroform/methanol (Ephritikhine et al. 2004): from all plant cell membranes analyzed, the envelope membranes contain the highest proportion of basic proteins.

4.2.3 *In silico* analyses

Bioinformatics approaches were also used to identify envelope membrane proteins (Koo and Ohlrogge 2002; Ferro et al. 2002, 2003; Rolland et al. 2003; Sun et al. 2004). For instance, Koo and Ohlrogge (2002) made attempts to predict plastid envelope proteins from the *Arabidopsis* nuclear genome by using computational methods and criteria such as the presence of N-ter plastid-targeting peptide (cTP) and of membrane-spanning domains (known thylakoid membrane proteins being subtracted). Using a combination of predictors and experimentally derived parameters, four plastid subproteomes, including envelope proteomes, were predicted from the fully annotated *Arabidopsis* genome by Sun et al. (2004). They were evaluated for distribution of physical-chemical parameters: they differ

strongly in average isoelectric point and protein size, as well as transmembrane distribution. Removal of the cleavable, N-terminal transit peptide sequences greatly affected isoelectric point and size distribution. Sun et al. (2004) observed that the Cys content was much lower for the thylakoid proteomes than for the inner envelope and related this observation to the role of the thylakoid membrane in light-driven electron transport.

Ferro et al. (2002) observed that chloroplast envelope transporters are synthesized as precursor proteins with a predicted cTP; they have a Res/TM ratio below 100; they contain more than 4 transmembrane domains; and have an isoelectric point above 8.8. These parameters were used to mine the AMPL database: Ferro et al. (2002) found that only 136 proteins (of the 25,498 predicted *Arabidopsis* proteins) meet all these requirements. About 35% of these proteins correspond to proteins belonging to plant transporter families, a few percent of these proteins are proteins involved in lipid or pigment metabolisms, whereas the remaining 50% are hypothetical proteins whose function could not be predicted on the basis of their primary structure. About 15% of these 136 proteins correspond to proteins identified by means of the proteomic approach (Ferro et al. 2002). This is very close to the137 membrane proteins probably targeted to plastids that were classified by Weber et al. (2005) as members of the transporter family.

5 Functions of chloroplast envelope proteins

Envelope proteins in Table 4 are classified among functional classes. Almost 25% of the proteins identified by proteomics are involved in lipid (fatty acids, glycerolipids, pigments and terpenoids) metabolism. Another 25% of the identified proteins are likely involved in metabolite and ion transport across the envelope, whereas more than 10% of the envelope proteome are either known components of the import machineries or homologous to them. In the following paragraphs, it is not our goal to address in detail all the envelope functions. For this, the readers are referred to all other chapters in this book that are specifically addressing the main aspects of the role of envelope membranes in plastid division, chloroplast nucleus communication, lipid trafficking and signaling, metabolite transport, etc. (see also Block et al. 2007). In this chapter, we will try to link the actual functions of envelope membranes with the proteins identified by Ferro et al. (2002, 2003) and Froehlich et al. (2003). Classification of proteins in given functional classes is also arbitrary. For instance some proteins involved in fatty acid or pigment metabolism are also involved in signaling, mechanosensitive (MscS) ion channels also play a role in controlling chloroplast size and shape and proteins in redox metabolism are often related to stress responses. One also should keep in mind that only putative functions of the proteins are indicated in Table 4. As these are based on available genome annotations and databases analysis, the actual function of a given protein can therefore be strikingly different. However, it is the link between the proteomic data and the actual envelope functions that provides insight into the roles of envelope membranes for the chloroplast and for the plant cell. This is of

special interest for controversial aspects of envelope metabolism that can only be addressed by protein identification.

5.1 Metabolism

5.1.1 Fatty acid and glycerolipid metabolisms

The question of a possible role of envelope membranes in the first steps of fatty acid biosynthesis was unexpected but was raised by the presence in envelope preparations of components of the plastid pyruvate dehydrogenase complex (ptPDC) and acetyl-CoA Carboxylase (ACCase) both considered as stromal enzymes. Table 4 shows that α (At1g01090) and β (At1g30120) subunits of E1, dihydrolipoamide dehydrogenase, two E2 isoforms (At3g25860, At1g34430) and E3 (At4g16155) from ptPDC are present in envelope preparations. This is somewhat puzzling. The plastid form of PDC provides acetyl-CoA and NADH for fatty acid biosynthesis (Mooney et al. 2002) and is sensitive to light/dark changes in the redox state of the chloroplast stroma (Tovar-Mendez et al. 2002). The presence of ptPDC components in envelope membranes (Table 4) raises the question of whether acetyl CoA formation (mostly for fatty acid synthesis) actually occurs in the vicinity of the envelope or whether this just reflects contamination by stroma. Ferro et al. (2003) identified E1 β subunit of ptPDC in NaCl washed envelope fractions, thus suggesting that it could be at least associated with the inner envelope membranes. Furthermore, the α, (At2g38040) and β (AtCg00500) subunits and the biotin carboxylase subunit (CAC2, At5g35360) of the heteromeric chloroplastic acetyl-coenzyme A carboxylase (ACCase) (which converts acetyl-CoA into malonyl-CoA) are also found in envelope preparation (Table 4). This is in agreement with a series of observations (Thelen and Ohlrogge 2002) suggesting that ACCase is anchored to the chloroplast envelope through non-ionic interactions with the carboxyltransferase subunits. Therefore, the presence of both ptPDC and ACCase in (or close to) inner envelope membrane could favour channelling of acetyl-CoA for its conversion into malonyl-CoA, an essential step in fatty acid biosynthesis.

Providing molecular evidence for previous biochemical studies showing that envelope membranes are the site of galactolipid, SQDG and PG synthesis (for reviews, Douce & Joyard, 1979, 1990; see also chapter by Andersson and Dörmann in this volume), envelope proteomics demonstrated the occurrence of all key enzymes for the biosynthesis of chloroplast glycerolipids (table 4): an enzyme of the Kornberg-Pricer pathway, 1-acylglycerol-3-phosphate O-acyltransferase (At4g30580), that catalyzes phosphatidic acid synthesis; of MGDG synthase (AtMGD1, At4g31780) and SQDG synthase (SQD2, At5g01220); and of at least three enzymes involved in PG synthesis, i.e. a CDP-diacylglycerol synthetase (At3g60620), and the two PG synthases, PGP1 (At2g39290) and PGP2

(At3g55030). In *Arabidopsis thaliana*, PG synthases are present in the three different compartments but are encoded by two related genes, *PGP1* and *PGP2* (Müller and Frentzen 2001). Although the presence of PGP1 in envelope membranes was expected, that of PGP2 in the envelope proteome is somewhat surprising since *PGP2* encodes the microsomal isozyme, whereas *PGP1* encodes a preprotein that is targeted to both plastids and mitochondria (Müller and Frentzen 2001; Babiychuk et al. 2003). PGP1 is essential for plastidial PG biosynthesis and for photoautotrophic growth, whereas it is redundant for the biosynthesis of PG in mitochondria (Frentzen 2004).

Proteomics also revealed a wide diversity of lipid metabolism enzymes: lipases (phospholipase, lysophospholipase, acyl hydrolases), a long-chain acyl-CoA synthetase (LACS9, see also Schnurr et al. 2002), two desaturases (omega-3 and omega-6 fatty acid desaturases), and enzymes involved in fatty acid hydroperoxide metabolism (lipoxygenase –LOX2, allene oxide synthase, allene oxide cyclase, and hydroperoxide lyase-like enzyme). Allene oxide synthase and hydroperoxide lyase are known envelope enzymes (Blée and Joyard 1996), they constitute a branch point leading specifically from 13(S)-hydroperoxy-9(Z), 11(E), 15(Z)-octadecatrienoic acid to 12-oxo-phytodienoic acid, the precursor of jasmonic acid.

Altogether, these results provide further evidence for the participation of chloroplast envelope membranes to several aspects of lipid metabolism (biosynthesis, transfer, desaturation, oxidation…) and in the production of lipid-derived plant growth regulators and defense compounds in response to extracellular stimuli.

5.1.2 Carotenoid, chlorophyll and prenylquinones metabolisms

The importance of chloroplast envelope membranes in the formation of isoprenoid chloroplast lipid constituents such as carotenoids, chlorophylls and quinones, previously raised by biochemical studies (for review see Douce and Joyard 1990), and often considered as controversial in the literature, was confirmed unambiguously by proteomics (see below).

Firstly, envelope proteomics detected several enzymes of carotenoid metabolism, such as phytoene dehydrogenase (At4g14210), ζ-carotene desaturase (At3g04870), carotene ε-ring hydroxylase (At3g53130), zeaxanthin epoxidase (At5g67030), and neoxanthin synthase (At1g67080) (Table 4). The two desaturases are on the common pathway leading to synthesis of lycopene, the precursor for xanthophylls (the main carotenoid group of chloroplasts). The carotene ε-ring hydroxylase is on the α-carotene branch of the pathway leading to the formation of lutein. The two other enzymes, zeaxanthin epoxidase and neoxanthin synthase, are on the branch leading to the formation of precursors of abscisic acid. Altogether, these results provide further support to a key role of envelope membranes in carotenoid biosynthesis, as proposed by Costes et al. (1979).

Secondly, several enzymes of chlorophyll biosynthesis are present in the envelope membranes (Table 4): protoporphyrinogene oxidase (At5g14220, At4g01690), Mg-protoporphyrin IX (Mg-Proto IX) monomethylester cyclase

(At3g56940), CHLM (Mg-Proto IX) methyltransferase (At4g25080), [3,8-DV]-Chlide a 8-vinyl reductase (At5g18660), and protochlorophyllide reductases (At4g27440, At1g03630). The dual localization of this biosynthetic pathway in the envelope and in thylakoids raised the question of the physiological significance of the envelope pathway. In fact, some of the intermediates formed in envelope may play a role in signalling between chloroplast and nucleus in order to coordinate chloroplast development and nuclear gene expression (see for instance Block et al. 2007). Indeed, envelope membranes contains key enzyme for the formation of one of the plastid signals, the chlorophyll intermediate Mg-Proto IX, that acts as a negative regulator of photosynthetic gene expression in the nuclei (Strand et al 2003). This function of Mg-Proto IX was confirmed by phenotypic analyses of a knock-out mutant and the possible implication of Mg-Proto IX methyl ester, the product of CHLM, in chloroplast-to-nucleus signalling was also suggested (Pontier et al. 2007). Furthermore, the FLU (for *flu*orescent) protein (At3g14110) was also identified in envelope membranes. This protein appears to regulate specifically the Mg^{2+} branch of the tetrapyrrole pathway and operates independently of heme (Meskauskiene et al. 2001). In *Chlamydomonas*, Flu-like proteins were shown to act as regulators of chlorophyll synthesis, and their expression is controlled by light and plastid signals (Falciatore et al. 2005). Therefore, owing to the presence of the chlorophyll biosynthetic pathway that release specific chlorophyll-derived signals, chloroplast envelope membranes play a key role in mediating the plastid-to-nucleus regulatory pathway (for reviews, Nott et al. 2006; Block et al. 2007; Chapter X).

Finally, envelope membranes also contain enzymes involved in the biosynthesis of prenylquinones. Although Soll et al. (1985) demonstrated that all enzymes involved in the last steps of α-tocopherol and plastoquinone-9 biosynthesis are localized on the inner envelope membrane; this has been a matter of debate (review by Block et al. 2007). Proteomic analyses (Table 4) demonstrate that, with the exception of 4-Hydroxyphenylpyruvate dioxygenase (HPPD), a cytosolic enzyme (Garcia et al. 1997), and tocopherol cyclase (VTE1) associated to plastoglobules (Vidi et al. 2006; Ytterberg et al. 2006), the other enzymes of prenylquinone biosynthesis were found in envelope membranes. The first committed reaction in prenylquinone biosynthesis is the condensation of homogentisic acid with prenyl (solanesyl, geranylgeranyl or farnesyl) diphosphate. AtHST (At3g1195) represents homogentisate solanesyltransferases involved in plastoquinone-9 biosynthesis (Sadre et al. 2006) and was localized in envelope membranes by proteomics (Table 4) and more recently by confocal microscopy (Tian et al. 2007). Overexpression of the enzyme in *A. thaliana* can affect tocopherol biosynthesis as well (Sadre et al. 2006). IEP37 (At3g63410, VTE3) is a major inner envelope membrane protein with a SAM-dependent methyltransferase activity (Teyssier et al. 1996), committed to the biosynthesis of plastid prenylquinones (Motohashi et al. 2003).

5.2 Transporters

Since early work on intact chloroplasts, the inner envelope membrane is known to represent the actual permeability barrier between plastids and the surrounding cytosol whereas the outer envelope membrane is expected to be freely permeable to small molecules owing to the presence of porins (see Weber et al. 2005). This is probably not as simple since substrate-specific gated pore-forming proteins were characterized in the outer envelope membrane (see Bölter and Soll 2001). Altogether, the combined proteomic and *in silico* approaches suggest that a series of known or putative transport systems are likely to be localized in the chloroplast envelope (Seigneurin-Berny et al. 1999; Ferro et al. 2002, 2003; Weber et al. 2005).

The major inner envelope proteins are triose-P/Pi translocators (reviewed by Weber et al. 2005), identified in all proteomic studies (Table 4). Such transporters are essential for controlling the Pi level in the stroma and the homeostasis required to initiate the Benson and Calvin cycle, especially during the dark/light and light/dark transitions. Interestingly, whereas some transporters, like the members of the triose-P/Pi, PEP/Pi, or Glucose-6P/Pi translocators catalyze an equimolar exchange of Pi, others like the putative H^+/Pi transporter could catalyze a net import of Pi to the chloroplast. Identification of these new phosphate transport systems in chloroplasts is expected to lead to a better understanding of their role in cell metabolism.

Envelope membranes contain two members of the 2-oxoglutarate/malate translocator family: DiT2-1 (At5g64290) and DiT1 (At5g12860). These proteins could differ in substrate specificity as DiT2-1was demonstrated to catalyze the transport of glutamate/malate (Renne et al. 2003). The identification of several other proteins is consistent with transport activities already associated with the chloroplast envelope. For example, several classes (belonging to at least two superfamilies) of amino acid transporters were identified in plants (Ortiz-Lopez et al. 2001). Identification of some members of the amino acid transporter families in envelope membranes (Table 4) provides some clues for distinguishing these transporters with overlapping substrate specificity. Several ABC proteins and NAP (non-intrinsic ABC protein) were also identified (Table 4). They are expected to play a role in the transport of a wide variety of substrates (metabolites, lipids, etc.) across the envelope. However, one of them, named STA1 (At5g58270), was expected to reside within mitochondria and was shown to be essential for transport of iron/sulfur clusters and iron homeostasis (Kushnir et al. 2001).

Several members of the Mitochondrial Carrier Family (MCF) were identified in envelope membranes (Table 4), many of them being only hypothetical proteins. In fact, not all MCF members are located in mitochondria: some are also present in peroxisomes, glyoxysomes and plastids (review by Haferkamp 2007) and their characteristic features are mostly structural (see Millar and Heazlewood 2003). Therefore the MCF denomination is misleading. In plants, MCF proteins are involved in the transport of solutes like nucleotides, phosphate, di- and tricarboxylates and therefore exhibit physiological functions similar to known isoforms from animal or yeast mitochondria. Interestingly, several MCF proteins mediate the

transport of different substrates like folates, S-adenosylmethionine, ADPglucose or ATP, ADP and AMP in plastids (see Chapter X). S-adenosylmethionine is formed exclusively in the cytosol but is imported to plastid where it plays a major role (Ravanel et al. 2004). A S-adenosylmethionine transporter was characterized by Bouvier et al. (2006) and is located in the chloroplast envelope, as previously suggested by bioinformatics (Koo and Ohlrogge 2002) and demonstrated by proteomics (Ferro et al. 2002, 2003). This transporter is probably dual targeted since it was also recently demonstrated to reside within mitochondria (Palmieri et al. 2006). Although the mitochondria were demonstrated to be the sole site of dihydrofolate synthesis in the plant cell, folate-mediated reactions were identified in the cytosol, the mitochondria, and the plastids (Ravanel et al. 2001), suggesting that folate must be imported into chloroplasts. Indeed, the AtFOLT1 protein (At5g66380), identified by bioinformatics as a candidate for folate transport across the chloroplast envelope (Ferro et al. 2002), was validated by Bedhomme et al. (2005). However, it has not yet been identified by proteomics and the exact function of the majority of the MCF proteins in *Arabidopsis* is still unknown.

Several ion channels were identified by envelope proteomics (Table 4). MSL proteins probably control plastid size and shape during plant development by altering ion flux across the envelope membranes (Haswell and Meyerowitz 2006; see above). KEA1 and KEA2 (At 1g01790, At4g 00630, respectively) are two of the putative K^+ efflux antiporters in *Arabidopsis* (Mäser et al. 2001). Two DMI1-like proteins are present in envelope membranes (At5g43745 and At5g02940) and they belong to the novel class of plastid-localized "Castor and Pollux" family that are absolutely required for early signal transduction events leading to endosymbioses (Imaizumi-Anraku et al. 2005). The *Arabidopsis* protein encoded by gene At5g49960, before the two DMI1-like proteins, is the most similar protein to *Medicago truncatula* proteins Castor and Pollux and DMI1. Therefore, one can question whether the two DMI1-like envelope proteins could play a role similar to that of DMI1. Imaizumi-Anraku et al. (2005) also raised the question of how are these proteins implicated in a process that starts with the perception of microbial signals at the plasma membrane and leads to changes in gene expression in the nucleus, among other responses.

Metal ions are essential for chloroplast development and function. Unfortunately, little is known about their transport across the chloroplast envelope. Envelope proteome shows several metal transporters (Table 4). For instance, AtMRS2-11 (At5g22830) is a Mg transporter of the corA group of bacterial Mg transporter family. It has been shown to complement a yeast double mutant and restores Al tolerance (Li et al. 2001). Several chloroplast P_{1B}-type ATPases are involved in metal ion transport across the envelope: in addition to PAA1 (Shikanai et al. 2003), HMA1 -that was identified by envelope proteomics (Ferro et al. 2003) - is also involved in Cu import into the chloroplast under high light conditions, thus allowing plants to respond to oxidative stress generated in these conditions (Seigneurin-Berny et al. 2006; see below).

Outer envelope membrane also contains substrate-specific gated pore-forming proteins (see Bölter and Soll 2001) and several of these high-conductance solute channels were identified by proteomics (Table 4): OEP21-1 (At1g76405), OEP24 (At3g52230), OEP24-II (At5g42960) and OEP37 (At2g43950). All of them have a

β-barrel structure. The OEP24 channels were the first outer envelope channel to be functionally characterized. They allow the flux of triose phosphate, dicarboxylic acids, positively or negatively charged amino acids, sugars, ATP, and Pi (Pohlmeyer et al. 1998). Hemmler et al. (2006) analyzed the Oep21 channel properties by biochemical and electrophysiological methods and proposed that it is involved in the regulation of the flux of phosphorylated carbohydrates, like 3-phosphoglycerate and glyceraldehyde 3-phosphate, across the outer membrane. OEP37 was reconstituted in vitro and was demonstrated to form a rectifying high conductance cation-selective channel (Goetze et al. 2006). OEP37 may constitute a novel peptide-sensitive ion channel in the outer envelope of plastids with function during embryogenesis and germination (Goetze et al. 2006).

5.3 Protein targeting: the protein import machinery of the chloroplast envelope

One of the key functions in chloroplast envelope membranes is the transport of nuclear-encoded proteins residing within the chloroplast. Numerous components of the chloroplast import machinery have been characterized, i.e. the translocon complexes at the outer (TOC complex) and inner (TIC complex) envelope membranes (for reviews see Hofmann and Theg 2005; Schleiff and Soll 2005; Kessler and Schnell 2006, Chapter X). Table 4 demonstrates that envelope preparations contain more than 20 proteins related to components of the protein import machinery. They can be classified into (a) known components of the TOC complex (Toc 33, Toc34, Toc 64, Toc75, Toc159); (b) known components of the TIC complex (Tic 20, Tic 21, Tic40, Tic55, Tic 110); (c) Tic20-like (IEP16), Tic55-like and Tic-62-like proteins; and (d) a series of OEP16-like proteins (HP15, HP22, HP30, HP30-2) with some homology to components of the mitochondrial inner membrane import machinery (Tim17/Tim22/Tim 23). To this list one should also add chaperones that are likely to be involved in protein import (see below).

Other envelope proteins identified through proteomic analyses are homologous to known components of the chloroplast or of the mitochondrial import machinery. The actual participation of these new proteins to protein import mechanisms into chloroplasts remains to be demonstrated. Are they true components of the TIC/TOC complexes or of distinct import system that could be present in chloroplast envelope membranes? Proteomic analyses led to the characterization of inner envelope proteins that do not use the classical TOC machinery for transport across the outer envelope membrane: ceQORH (At4g13010) that was devoid of cleavable *N*-terminal transit sequences and contained internal targeting information (Miras et al. 2002, 2007) and Tic32, which contained an N-terminal but uncleavable presequence (Nada and Soll 2004). Finally, Villarejo et al. (2005) recently showed that a chloroplast-located protein takes an alternative route through the secretory pathway, and becomes N-glycosylated before entering the chloroplast. Therefore, the actual components of these new import machineries need to be identified.

5.4 Envelope proteins and lipid trafficking

Lipid trafficking between the chloroplast and the endomembrane system and within plastids are key processes in plant development (reviews by Benning et al. 2006; Jouhet et al. 2007). Proteomic data only provide few elements in understanding the processes (Table 4). Firstly, the long-chain acyl-CoA synthetase (At1g77590) could be responsible for the export of chloroplast-synthesized fatty acids to the endomembrane system (two other proteins encoded by At3g23790 and At4g14070 genes are also annotated as acyl-CoA synthetases). Secondly, glycerolipid synthesis requires that the eukaryotic type of DAG backbone, necessary for the formation of eukaryotic chloroplast glycerolipids, comes from endomembrane glycerolipids. The TGD protein (At3g20320) in the chloroplast envelope proteome promises new insights into lipid-trafficking between the envelope and the endomembrane system (review by Benning et al. 2006) Thirdly, thylakoids formation requires a massive transfer of the different lipids synthesized in the envelope to the thylakoids and selective lipid transport from the inner envelope membrane to the thylakoids has been demonstrated (Rawyler et al. 1995). This process might involve VIPP1 (Vesicle Inducing Protein in Plastids 1, encoded by At1g65260) and/or THF1 (THYLAKOID FORMATION 1 protein, encoded by At2g20890), proteins involved in thylakoids formation through vesicular trafficking. The intriguing point is that THF1 is localized to both the outer envelope membrane and the stroma and is supposed to interact with GPA1, a Plasma Membrane G-Protein, in a putative sugar-signaling mechanism that would be essential for thylakoid formation (Huang et al. 2006 and references therein).

5.5 Protein degradation and regeneration (chaperone and protease)

In *Arabidopsis*, plastids contain more than 11 types of proteases encoded by more than 50 genes (Sakamoto 2006). Froehlich et al. (2003) and, to a lesser extent, Ferro et al. (2003) identified series of HSP70, ATP-dependent ClpP and ClpR as well as proteases of the GTP dependent FtsH family in envelope preparations (Table 4). Of the 15 plastid-localized Clp proteolytic proteins (Adam et al. 2006), about 10 were found in envelope preparations (ClpS1, ClpC1-6 and ClpR2-4). In addition, FtsH2, 5, 7 were identified in envelope proteomes (Table 4). Such proteases are involved in numerous aspects of the biogenesis and maintenance of chloroplasts, such as the removal and degradation of signal sequences, the degradation of partially assembled complexes or damaged proteins, adaptation to changes in environmental conditions, and the breakdown of chloroplasts in senescing leaves (Adam 2005). It is generally assumed that most of these proteases reside within the stroma; however, some of them are associated with envelope-specific processes. For instance, ClpC1 (HSP 100), the first protease demonstrated as being associated with the chloroplast protein translocation machinery in the inner envelope (see for instance Nielsen et al. 1997), was indeed identified by pro-

teomics (Table 4). It is possible that the diverse set of envelope-bound Clp found in proteomic studies is required for the translocation of many proteins across the chloroplast envelope membranes, presumably by providing energy in the form of ATP hydrolysis for protein precursor translocation across the envelope membranes.

5.6 Plastid division and positioning

Proteins involved in chloroplast division were identified by searching possible homologues of components of known prokaryotic division machinery (e.g. FtsZ, MinD, MinE and GC1/SulA), by detailed analyses of the *arc* (accumulation and replication of chloroplasts) mutants, and of components that affect chloroplast division but were not directly involved in the division machinery (MscS-like or MSL proteins) (for reviews see Aldrige et al 2005, Maple and Møller 2006, Chapter X). Proteomic studies identified some of these proteins in envelope preparations (Froehlich et al. 2003; Table 4). For instance, ARC6 (homologous to a bacterial cell division protein), which was identified from *arc6* mutants that contain only one giant chloroplast per cell (Vitha et al. 2003) and GC1 (for Giant Chloroplast1), which also has a prokaryotic origin (Maple et al. 2004). Two MSL proteins in *Arabidopsis thaliana* envelope membranes, MSL2 and MSL3 (Haswell and Meyerowitz 2006), are related to MSL1 (At4g00290), which was identified by proteomics (Table 4). In the model proposed by Haswell and Meyerowitz (2006), MSL2 and MSL3 control plastid size, shape, and perhaps division by altering ion flux in response to changes in membrane tension.

Table 4 also shows the presence of a CHUP1-like protein (At1g07120). CHUP (chloroplast unusual positioning) proteins are required for organellar positioning and movement in plant cells and were identified from the screening of *Arabidopsis* mutants defective in chloroplast photorelocation movement (Oikawa et al. 2003, Chapter X). Proteomics also revealed TH65 kinesin related protein (At5g10470) and a kinesin-like protein (Xklp2-like protein similar to microtubule associated protein, At1g03780) in envelope preparations. Functional studies are necessary to determine whether such proteins are actually motor proteins that could transport chloroplasts along microtubule tracks. Like CHUP1, they exhibit coiled-coil domains that are expected to play a role in subcellular infrastructure maintenance and trafficking control (Rose et al. 2005).

5.7 Signaling

A key role for envelope membranes is the production of lipid- and pigment-derived signaling molecules (see above). For instance, envelope membranes contain the whole set of enzymes that convert polyunsaturated fatty acids into jasmonate precursors; the cleavage of xanthophylls formed in envelope membranes is re-

sponsible for the formation of abscisic acid envelope-synthesized chlorophyll precursors mediate plastid-to-nucleus signaling.

Several envelope proteins are probable candidates as components of cellular signaling pathways. Two homologous hypothetical proteins (AtCDF1-like proteins, encoded by At3g51140 and At5g23040 genes) were identified in envelope membranes (Table 4). Interestingly, Kawai-Yamada et al (2005) demonstrated that overexpression of *Cdf1* gene (At5g23040) of *Arabidopsis* in yeast could induce apoptosis-like cell death in yeast but here, CDF1 fused to GFP was expressed in mitochondria.

Furthermore, several envelope proteins contain putative domains that are present in components of signaling pathways, such as the protein encoded by gene At5g64430 with a octicosapeptide/Phox/Bem1p (PB1) domain (Moscat et al. 2006). In addition, the identification in envelope membranes of two DMI1-like proteins (Table 4), putative members of the "Castor and Pollux" family of proteins that is required for early signal transduction events leading to endosymbioses, clearly opens a new field of research (see above).

5.8 Stress and redox metabolism

Plants are submitted to a wide variety of environmental stresses, like high light, drought, nutrient and temperature that can induce oxidative stress. In addition, intermediates in electron transfer reactions may react with other cell constituents and produce radicals and reactive oxygen species. Reactive oxygen species are responsible for major damages to membrane lipids and proteins. Consequently, an efficient antioxidant network is essential for protecting cell functions, particularly in chloroplasts. A whole set of metabolites such as glutathione and ascorbate, carotenoids, tocopherols and enzymes such as superoxide dismutase (SOD), ascorbate peroxidase (APX) and glutathione peroxidase are known elements of antioxidant metabolism (for review, see Mullineaux and Karpinski 2002). The presence of α-tocopherol and xanthophylls in envelope membranes (see above) is a first element for this system to play a role in oxidative stress responses. Furthermore, several proteins that could be involved in oxidative stress responses were identified in *Arabidospis* envelope membranes (Table 4): namely a phospholipid hydroperoxide glutathione peroxidase (GPX2, At2g25080) and an ascorbate peroxydase (APX3, At4g35000). Two members of the peroxiredoxins (Prx, for review see Dietz, 2007) class of enzymatic antioxidants, PrxA (At3g11630) and PrxQ (At3g26060), were also identified (Table 4). However, PrxQ was also assigned to be bound to thylakoids (Lamkemeyer et al. 2006). In fact, most of these proteins do not have any transmembrane domains and are probably bound to, and active at, the stromal surface of the envelope membranes.

The stromal Cu/Zn SOD contains Cu, which is toxic at high concentration, due to generation of oxygen and hydroxyl free radicals and the oxidation of dithiols to disulfide in proteins (see for instance Shingles et al. 2004). Indeed, Seigneurin-Berny et al. (2006) demonstrated that *Arabidopsis* plants with a mutation in the

gene encoding the envelope protein HMA1, a member of the metal-transporting P1B-type ATPases family (see above), have a photosensitivity phenotype under high light. This demonstrates that Cu homeostasis in chloroplast, and therefore Cu transport across the envelope, is essential for an efficient photoprotection, especially under high light.

One original way to prevent photodamage in chloroplasts when plants are exposed to high light levels could be chloroplast avoidance movement, in which chloroplasts move from the cell surface to the periphery of cells under high light conditions. Kasahara et al. (2002) have shown that *Arabidopsis chup1* mutants (devoid of CHUP1 envelope protein, see above) are defective in chloroplast avoidance movement and are therefore more susceptible to damage in high light than wild-type plants.

Finally, several envelope (or envelope-bound) proteins are induced upon stress. For example, GLX1 (At1g11840) and ERD4 (At1g30360) are induced upon drought (Seki et al. 2001), and the damages caused to membrane proteins submitted to an oxidative stress require the presence of active repair mechanisms, such as chaperones and proteases (see above).

6 Conclusion

Our understanding of the essential role of chloroplast envelope membranes in the plant cell is strongly improving. As discussed by Sun et al. (2004) "the assembly of rigorously curated subcellular proteomes is in itself also important as a parts list for plant and systems biology". The picture emerging from our present understanding of chloroplast envelope membranes is that of a major node for integration of metabolic and ionic networks in plant cell metabolism and of a key player in chloroplast biogenesis and signaling for the co-ordinated gene expression of chloroplast-specific protein. Furthermore, the lipid and protein constituents of chloroplast envelope membranes reflect both their prokaryotic and eukaryotic origin.

Finally, one should keep in mind that the protein list in Table 4 is only a snapshot at a given time: more genuine envelope proteins are expected to be identified with the increasing number of proteome analyses performed by different groups combined to recent and fast technological developments in mass spectrometry.

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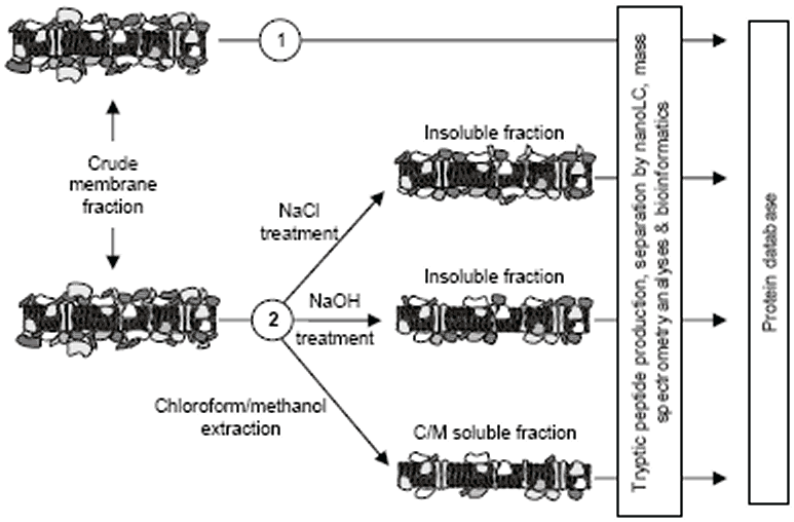

Fig. 1. Strategies for envelope proteomics. Scheme 1: Froehlich et al. (2003) directly used envelope preparation without any fractionation together with off-line multidimensional protein identification technology (off-line MUDPIT) to analyze the envelope proteome. Scheme 2: Ferro et al. (2002, 2003) analyzed envelope proteins from spinach and *Arabidopsis* chloroplasts by using a set of complementary methods, i.e. solubilization in chloroform/methanol, and alkaline and saline treatments, prior mass spectrometry analyses.

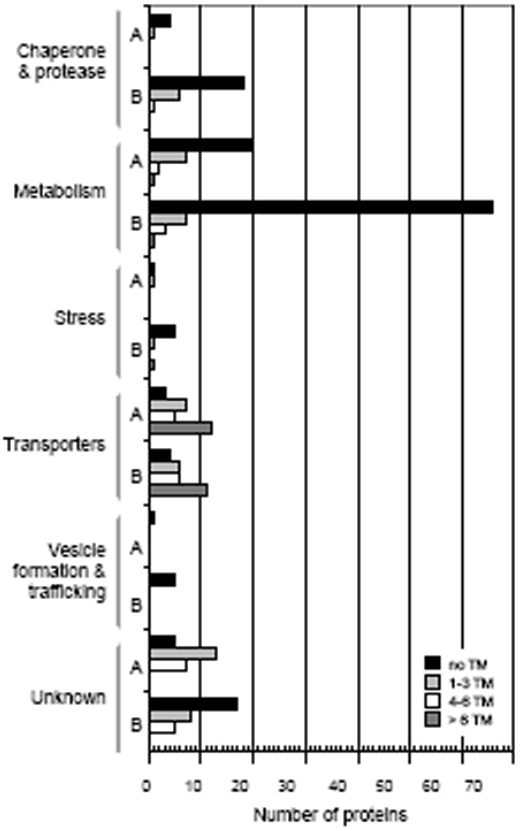

Fig. 2. Functional classification of envelope proteins identified by proteomics. The number of putative transmembrane domains is indicated for each protein class. A: data from Froehlich et al. (2003): B: data from Ferro et al. (2002, 2003).

T**able 1**. Fatty acids present in a mixture of total envelope lipids and individual components (Siebertz et al. 1979)

	16:0	16:1	3*t*-16:1	16:2	16:3	18:0	18:1	18:2	18:3	20:0	
	Mol/100 mol										% of total
Total	14.0	0.4	3.6	trace	9.3	0.6	3.9	8.0	59.9	20.0	-
MGDG	5.2	0.7	0	trace	17.3	1.2	2.3	3.2	68.9		13.2
DGDG	13.1	0.8	trace	0.3	5.1	1.7	5.1	5.9	68.0		21.6
SQDG	44.0	trace	0	0	1.8	1.2	1.9	5.0	46.1		7.5
PG	17.9	0.7	33.6	0	0.5	1.0	2.0	4.4	39.3	0.9	7.3
PC	20.5	1.3	trace	trace	0.5	1.5	13.2	25.9	36.8	0.3	16.5
Unknown	31.6	6.7	0	0	1.2	4.5	10.7	16.5	28.7		1.3
PE	30.7	7.2	0	0	0	4.5	13.3	18.4	25.9		trace
TAG	5.5	0.6	0.5	trace	13.7	4.7	9.0	12.6	54.5		trace
DAG	5.7	0.8	1.0	trace	15.2	0.9	2.9	3.7	69.9		16.8
TGDG	4.8	2.2	0	0	18.8	0.7	2.3	1.7	69.6		5.2
TeGDG	8.8	2.0	0	0	23.1	3.9	4.4	1.9	55.9		2.1
acylated DGDG	9.7	0	0	0	17.5	1.3	3.4	2.5	64.3		1.8

Lipids were extracted from purified spinach chloroplast envelope membranes containing 122 mg of dried lipids. Lipid proportions are given as mol/100 mol in the last column. Fatty acids are characterized by a number of carbon atoms and double bonds. 16:1 includes *cis*-7 and *cis*-9 isomers. The unknown phospholipid has chromatographic properties similar to PI. For detailed structures of all plant galactolipids and glycolipids molecular species the reader is referred to Heinz (1996) where they have been described and discussed in detail. TGDG, TeGDG and acyl DGDG are “artificial lipids” formed during the course of envelope preparation (see Table 2). Abbreviations: TAG, triacylglycerol; DAG, diacylglycerol; MGDG, monogalactosyldiacylglycerol; DGDG, digalactosyldiacylglycerol; TGDG, trigalactosyldiacylglycerol; TeGDG, tetragalactosyldiacylglycerol; SQDG, sulfoquinovosyldiacylglycerol; PG, phosphatidylglycerol; PC, phosphatidylcholine; PE, phosphatidylethanolamine.

Table 2 Lipid composition of envelope membranes from non-treated, thermolysin-treated and phospholipase C-treated intact spinach chloroplasts.

Lipids	Thermolysin-treated	Non-treated	Phospholipase C-treated
MGDG	38	13.5	16
DGDG	30	33.5	33
TGDG	0	3	4
TeGDG	0	1.5	2
SQDG	7	7.5	7
PG	9	9	7
PC	13.5	15	trace
PI	2	3	3
PE	0	0	
DAG	>0.1	13.5	26

Data from thermolysin-treated chloroplasts and from phospholipase-C treated chloroplasts are from Dorne et al. (1982) and Dorne et al. (1990), respectively. It was shown that thermolysin as well as phospholipase C do not penetrate the outer envelope membranes (in the experimental conditions used), and could be used to probe protein and lipid components of the outer leaflet of the outer envelope membranes. Note that envelope membranes from thermolysin-treated intact chloroplasts are devoid of TGDG, TeGDG and DAG, and contain more MGDG than DGDG, the phospholipid content is close to that of the control. In phospholipase-C treated intact chloroplasts, there is no PC but large amounts of DAG, in contrast, the galactolipid content is almost identical to that of the control. The thermolysin experiment indicates that a mild proteolytic digestion of the outer surface of the chloroplast envelope hydrolyzes a galactolipid-metabolising outer envelope protein: this enzyme (GGGT) converts MGDG into DGDG, TGDG, TeGDG and DAG. This suggests that the genuine envelope lipid composition is close to that from thermolysin-treated chloroplasts. Phospholipase C-treatment (which hydrolyses PC and forms DAG) indicates that PC is accessible from the cytosolic surface of the envelope, and therefore concentrated in this leaflet of the outer envelope membrane. Numbers are % of total lipids.

Table 3 Distribution of lipid compounds in chloroplasts

	total envelope membranes	outer envelope membrane	inner envelope membrane	thylakoids
Total polar lipids[a] (mg /mg protein)	1.2 - 1.5	2.5 - 3	1	0.6 - 0.8
Polar lipids (% of total)				
MGDG	32	17	55	57
DGDG	30	29	29	27
SQDG	6	6	5	7
PC	20	32	0	0
PG	9	10	9	7
PI	4	5	1	1
PE	0	0	0	0
Total Chlorophylls[b] (µg /mg protein)	0.1 - 0.3	nd	nd	160
Chlorophylls (% of total in the fraction)				
Chlorophyll a	86	nd	nd	72
Chlorophyll b	14	nd	nd	28
Chlorophyll precursors[b] (Protochlorophyllide + Chlorophyllide, µg /mg protein)	0.41	nd	nd	0 - 0.35
Total Carotenoids[c] (µg /mg protein)	6 - 12	2.9	7.2	20
Carotenoids (% of total)				
β-Carotene	11	9	12	25
Violaxanthin	48	49	47	22
Lutein + Zeaxanthin	21	16	23	37
Antheraxanthin	6	-	5	-
Neoxanthin	13	26	13	16
Total Prenylquinones[d] (µg /mg protein)	4 - 11	4 - 12	4 - 11	4 - 7
Prenylquinones (% of total)				
α-Tocopherol + α-Tocoquinone	69	81	67	24
Plastoquinone-9 + Plastoquinol	28	18	32	70
Phylloquinone K1	3	1	1	6

Data are average values from spinach and corrected (for polar lipids) from thermolysin-experiments (see Table 2). Adapted from Block et al. (2007). nd, not determined.

Table 4. The chloroplast envelope proteome.

This protein list is derived from data published by Ferro et al. (2002, 2003) and Froehlich et al (2003). It is a "curated" list: we have removed from published envelope proteomes those proteins we suspect to be contaminants (see Figure 2). This was performed manually for each protein identified in envelope fractions by searching in databases (see for instance Schwacke et al. 2004, Friso et al. 2004), in published protein lists from various origin and in proteomic analyses we (and others) performed on different chloroplast subfractions (especially stroma) prepared from Arabidopsis chloroplasts (to be published). Target P was used to determine where the protein was targeted (C: chloroplast; M: mitochondria). Various informations (i.e. presence of a chloroplast transit peptide cTP, the calculated gravy index, Mw and Pi are from PPDB (a Plant Proteome DataBase for *Arabidopsis thaliana* and *Zea mays*; Friso et al. 2004). The number of transmembrane domains (TM) was determined using Aramemnon (Schwacke et al. 2003). This protein list is only a snapshot at the time of publication: more genuine envelope proteins are expected to be identified with the increasing number of proteome analyses performed by different groups combined to recent and fast technological developments in mass spectrometry.

Gene	Description	Ferro et al. (2002, 2003)	Froelich et al. (2003)	TargetP	cTP	TM	Calc. gravy	Calc. MW (kD)	Calc. PI
Metabolism (lipid)									
At1g01090	PDH (E1) ALPHA E1 alpha subunit, pyruvate DH complex, chloroplast precursor	-	+	C	Y	ND	-0,269	47,17	7,16
At1g30120	PDH (E1) BETA (HP44b) E1 beta subunit, pyruvate DH complex, chloroplast precursor (EC 1.2.4.1)	+	-	C	Y	ND	-0,093	44,24	5,91
At3g25860	LAT2 (E2) Dihydrolipoamide acetyltransferase, pyruvate DH complex, chloroplast precursor	-	+	C	Y	ND	0,045	50,08	8,33
At1g34430	LTA2 (E2, EMB3003) Dihydrolipoamide acetyltransferase, pyruvate DH complex, chloroplast precursor	-	+	C	Y	ND	0,13	48,3	8,8
At4g16155	PTLPD2 (E3) Dihydrolipoamide dehydrogenase 2, pyruvate DH complex, chloroplast precursor	-	+	C	Y	ND	0,026	60,14	7,29
At2g38040	ACCDa (HP88b, CAC3) Acetyl-CoA carboxylase alpha chain, chloroplast precursor	+	+	C	Y	ND	-0,524	85,3	5,61
AtCg00500	ACCDb Acetyl-CoA carboxylase beta chain, chloroplast encoded	+	-	C	N	ND	-0,402	55,61	5,89
At5g35360	CAC2 Acetyl-CoA carboxylase, biotin carboxylase subunit	-	+	C	Y	ND	-0,135	58,38	6,84
At4g25050	ACP4 ACP-like acyl carrier-like protein, chloroplast precursor	-	+	C	Y	ND	-0,013	14,54	4,73
At1g77590	LACS9 (HP76) Long-chain-fatty-acid-CoA ligase family protein, chloroplast precursor	+	+	C	N	ND	-0,064	76,17	6,53
At3g23790	Long-Chain Acyl-CoA Synthetase, chloroplast precursor	-	+	C	Y	ND	-0,229	81,14	8,73
At4g14070	HP81 (AAE15) Long-Chain Acyl-CoA synthetase, chloroplast precursor	+	+	C	Y	ND	-0,219	81,46	8,89
At3g11170	FAD3C (FAD7, FADD) omega-3 fatty acid desaturase, chloroplast precursor	+	+	C	Y	4	-0,299	51,17	8,14
At4g30950	FAD6C (FADC) Omega-6 fatty acid desaturase, chloroplast precursor	+	+	C	Y	3	-0,07	51,22	9,01

At4g30580	LPAT1 (LPAAT, ACT2) Lysophosphatidic 1-acylglycerol-Phosphate acyltransferase, chloroplast precursor	+	-	C	N	3	0,029	39,39	9,87
At4g31780	MGD1 (MGDA) monogalactosyldiacylglycerol synthase, chloroplast precursor	+	+	C	Y	ND	-0,157	58,53	9,29
At5g01220	SQD2 sulfolipid synthase/UDP-sulfoquinovose:DAG sulfoquinovosyltransferase, chloroplast precursor	-	+	C	Y	1	-0,108	56,63	8,61
At3g60620	CDP-Diacylglycerol synthetase, chloroplast precursor	-	+	C	N	7	0,336	43,25	9,3
At2g39290	PGPS1 (HP32c, PGP1) phosphatidylglycerolphosphate synthase, dual targeted	+	-	C	Y	2	0,224	32,15	10,3
At3g55030	PGPS2 (HP25b, PGP) phosphatidylglycerolphosphate synthase	+	-	-	Y	4	0,458	25,22	9,61
At3g10840	Lysophospholipase (EC 3.1.1.5), hydrolase, alpha/beta fold family protein	-	+	C	N	ND	-0,053	50,88	8,59
At1g52570	PLDA2 (PLD2) phospholipase D alpha 2 (EC 3.1.4.4)	-	+	-	N	ND	-0,408	91,59	5,82
At1g33810	GDSL-lipase 1, GDSL-motif lipase, hydrolase family protein	-	+	-	N	ND	-0,781	15,69	8,82
At3g14210	GDSL-like Lipase, Acylhydrolase like protein (ESM1)	-	+	S	N	ND	-0,145	44,06	7,58
At1g07420	SMO2 sterol 4-alpha-methyl-oxidase 2	-	+	S	N	4	0,098	30,92	8,63
At3g25760	AOC1 (ERD12-1) Allene oxide cyclase 1, chloroplast precursor	-	+	C	Y	ND	-0,265	27,8	9,11
At3g25770	AOC2 (ERD12-2) Allene oxide cyclase 2, chloroplast precursor	-	+	C	Y	ND	-0,231	27,63	6,9
At5g42650	CP74A (AOS) Allene oxide synthase, chloroplast precursor	+	+	C	Y	ND	-0,231	58,19	8,75
At4g15440	HPL-like hydroperoxide lyase-like protein	-	+	-	N	ND	-0,077	42,66	5,64
At5g16010	3-oxo-5-alpha-steroid 4-dehydrogenase (steroid 5-alpha-reductase) family protein	-	+	-	N	6	0,446	30,12	9,37
At3g45140	LOX2 Lipoxygenase, chloroplast precursor (1.13.11.12)	-	+	C	Y	ND	-0,465	102,04	5,42
Metabolism (vitamins & pigments...)									
At4g14210	CRTI (PDS3) Phytoene dehydrogenase, chloroplast precursor (EC 1.14.99.-)	-	+	C	Y	ND	-0,135	62,96	6,07
At3g53130	LUT1 Carotenoid epsilon-ring hydroxylase, chloroplast precursor, Cytochrome P450-like	-	+	C	Y	ND	-0,181	60,55	6,04
At3g04870	ZDS1 (ZCD) Zeta-carotene desaturase, chloroplast precursor (EC 1.14.99.30)	-	+	C	Y	ND	-0,109	61,63	7,03
At5g67030	ZEP (LOS6, ABA1) Zeaxanthin epoxidase, chloroplast precursor	-	+	C	Y	ND	-0,295	73,84	6,6
At3g09580	HP52b (PDS-like) weak similarity to phytoene dehydrogenase	+	-	C	Y	ND	0,005	52,25	7,02
At3g14110	FLU protein, tetratricopeptide repeat (TPR)-containing protein	-	+	C	N	1	-0,311	34,58	9,09
At5g18660	DVR [3,8-DV]-Chlide a 8-vinyl reductase	-	+	C	Y	ND	-0,101	45,89	7,47
At3g56940	CHL27 (CRD1, AT103) magnesium-protoporphyrin IX monomethylester [oxidative] cyclase	-	+	C	Y	ND	-0,348	47,63	8,55
At5g14220	PPOII (PPOX, HEMG2/MEE61) protoporphyrinogen IX oxidase	-	+	-	N	ND	-0,303	55,63	8,35
At4g01690	PPOC (PPOX) protoporphyrinogen oxidase, chloroplast precursor (EC 1.3.3.4)	-	+	M	Y	1	-0,131	57,69	9,13
At4g25080	CHLM Magnesium-protoporphyrin IX methyltransferase, chloroplast precursor (EC 2.1.1.1)	-	+	C	Y	ND	-0,055	33,79	7,68
At4g27440	PORB Protochlorophyllide reductase B, chloroplast precursor	+	+	C	Y	ND	-0,198	43,36	9,22
At1g03630	PORC Protochlorophyllide reductase C, chloroplast precursor	-	+	C	Y	ND	-0,351	43,88	9,18

At3g11950	AtHST (HP43) homogentisate solanesyl- geranylgeranyl-farnesyl-transferase, chloroplast precursor	+	-	-	Y	7	-0,155	106,65	5,15
At3g63410	VTE3 (APG1, IEP37) SAM-dependent methyl transferase involved in ubiquinone, menaquinone biosynthesis	+	+	C	Y	1	-0,268	37,92	9,18
Metabolism (others)									
At3g60750	TKL-1 (TKTC) transketolase-1, chloroplast precursor	+	-	C	Y	2	-0,238	79,96	5,93
At3g01500	CAHC (CA1) beta carbonic anhydrase 1, chloroplast precursor (EC 4.2.1.1)	+	+	-	N	ND	-0,025	29,5	5,53
At5g14740	CA2 (CAH2) beta carbonic anhydrase 2, chloroplast precursor (EC 4.2.1.1)	-	+	-	Y	ND	-0,073	28,34	5,36
At1g74640	conserved plant and cyanobacterial, hydrolase, alpha/beta fold family protein, similar to Slr1235 protein [Synechocystis sp]	-	+	C	Y	ND	-0,221	41,05	8,3
At5g38520	hydrolase, alpha/beta fold family protein, low similarity to hydrolase	-	+	C	Y	ND	0	39,57	6,54
At3g06510	HP70 (SFR2) glycosyl hydrolase family 1 protein, similar to beta-galactosidase	+	+	S	N	1	-0,258	70,77	8,79
At5g35170	AK adenylate kinase, chloroplast precursor (EC 2.7.4.3)	-	+	C	Y	ND	-0,307	65,73	8,84
At1g10510	HP73 (EMB2004) similar to ribonuclease inhibitor, contains Leucine Rich Repeat domains	+	+	C	Y	1	-0,134	64,72	7,18
At3g63170	weak similarity to Chalcone isomerase	-	+	C	Y	ND	-0,118	30,39	6,97
At5g39410	SCPDH Probable mitochondrial saccharopine dehydrogenase family (EC 1.5.1.9)	-	+	-	Y	ND	-0,178	49,68	8,53
Plastid division and positioning									
At5g42480	ARC6 (DNAJ, FTN2) plastid division protein, chloroplast precursor	-	+	C	Y	2	-0,157	88,26	4,76
At2g21280	GC1 plastid division protein (Giant Chloroplast 1), chloroplast precursor	-	+	C	Y	ND	0,022	37,74	9,31
At5g10470	TH65 kinesin motor protein-related TH65 protein	-	+	C	Y	ND	-0,403	141,03	5,86
At1g07120	CHUP1-like Chloroplast Unusual Positioning, essential for Proper Chloroplast Positioning	-	+	-	N	ND	-0,891	44,93	9,28
At1g03780	Xklp2-like, similar to microtubule-associated protein	-	+	-	N	ND	-0,846	78,65	9,22
Protein degradation and regeneration (protease & chaperone)									
At5g02500	HSP70-1 (HSC71) Heat shock cognate 70 kDa protein 1	-	+	-	N	ND	-0,436	71,35	5,02
At1g08640	DNAJ domain containing protein	-	+	C	Y	3	-0,072	32,9	9,82
At2g30950	FtsH2 (VAR2) protease, chloroplast precursor	-	+	C	Y	1	-0,141	74,15	5,99
At3g16290	FtsH protease family protein	-	+	C	N	2	-0,484	99,86	9,27
At3g47060	FtsH7 protease, chloroplast precursor	+	-	C	Y	2	-0,257	87,8	8,3
At5g42270	FtsH5 (FtsH2, VAR1) cell division protease, chloroplast precursor (EC 3.4.24.-)	-	+	C	Y	ND	-0,116	75,23	5,36
At5g64580	AAA-type ATPase family protein, similar to zinc dependent protease	-	+	C	Y	1	-0,359	96,85	5,66
At3g04340	FtsH (EMB2458) protease family protein	-	+	-	-	ND	-0,325	111,01	8,01
At5g35210	SREBP-like peptidase M50 family protein	-	+	-	N	4	-0,289	174,28	6,48

At4g25370	ClpS1 Clp amino terminal domain-containing protein, chloroplast precursor	-	+	C	Y	ND	-0,273	26,05	9,24
At4g24280	cpHSP70-1 (DnaK homologue) heat shock protein 70-1, chloroplast precursor	-	+	C	Y	ND	-0,32	76,5	5,06
At5g49910	cpHSP70-2 (Dnak homologue) heat shock protein 70-7, chloroplast precursor	-	+	C	Y	ND	-0,358	76,99	5,17
At5g50920	ClpC1 (Hsp100) ATP-dependent Clp protease ATP-binding subunit, chloroplast precursor	+	+	C	Y	ND	-0,394	103,45	6,36
At3g48870	ClpC2 (Hsp93-III) ATP-dependent Clp protease ATP-binding subunit	-	+	C	N	ND	-0,313	105,77	6,06
At1g66670	ClpP3 (nClpP3) ATP-dependent Clp protease proteolytic subunit, chloroplast precursor	-	+	C	Y	ND	-0,313	33,92	7,59
At5g45390	ClpP4 (nClpP4) ATP-dependent Clp protease proteolytic subunit, chloroplast precursor	-	+	C	Y	ND	-0,043	31,49	5,37
At1g02560	ClpP5 (nClpP1) ATP-dependent Clp protease proteolytic subunit, chloroplast precursor	+	+	C	Y	ND	-0,197	32,35	8,34
At1g11750	ClpP6 (nClpP6) ATP-dependent Clp protease proteolytic subunit, chloroplast precursor	-	+	C	Y	ND	-0,128	29,38	9,37
At1g12410	ClpR2 (nClpP2) ATP-dependent Clp protease proteolytic subunit, chloroplast precursor	+	+	C	Y	ND	-0,428	31,2	9,19
At1g09130	ClpR3 (nClpP8) ATP-dependent Clp protease proteolytic subunit, chloroplast precursor	-	+	C	Y	2	-0,145	36,3	8,63
At4g17040	ClpR4 (nClpP9) ATP-dependent Clp protease proteolytic subunit, chloroplast precursor	-	+	C	Y	ND	-0,207	33,44	9,33
At4g12060	ClpS2 Clp amino terminal domain-containing protein	-	+	C	Y	ND	-0,166	26,56	8,85
At3g13470	Cpn60-beta-1 chaperonin, similar to RuBisCO subunit binding-protein beta subunit, chloroplast precursor	-	+	C	Y	ND	-0,056	63,34	5,59
At5g20720	CH10C (Cpn21, Cpn20, CH1C) chloroplast Cpn21 chaperonin	+	+	C	Y	ND	-0,082	26,8	8,85
At3g62030	CP20C (ROC4) peptidyl-prolyl cis-trans isomerase, chloroplast precursor	-	+	C	Y	ND	-0,169	28,2	8,83
Protein modification									
At1g02980	CUL2 cullin family protein, similar to cullin 1 from Homo sapiens	-	+	-	N	ND	-0,402	85,96	7,26
At1g76370	APK1A -like putative protein kinase, similar to protein kinase APK1A	-	+	-	N	ND	-0,284	42,31	9,25
Protein targeting									
At5g28750	Tha4 mttA/Hcf106 family, similar to thylakoid assembly 4 protein, chloroplast precursor	-	+	C	Y	1	-0,26	15,71	9,22
At4g03320	Tic20-IV subunit of the translocon at the inner envelope membrane, chloroplast precursor	-	+	C	Y	4	0,155	32,52	9,75
At4g33350	Tic22 (HP27) subunit of the translocon at the inner envelope membrane, chloroplast precursor	+	+	C	Y	ND	-0,36	30,1	9,31
At5g16620	Tic40 (PDE120) translocon Tic40-like protein, chloroplast precursor	+	+	C	Y	1	-0,55	48,9	5,37

At2g24820	Tic55 subunit of the translocon at the inner envelope membrane, Rieske (2Fe-2S) domain-containing protein, chloroplast precursor	+	+	C	Y	2	-0,281	60,6	8,94
At1g06950	Tic110 (HP112) subunit of the translocon at the inner envelope membrane, chloroplast precursor	+	+	C	Y	2	-0,3	112,12	5,72
At2g47840	Tic20-like (IEP16) low similarity to Tic20, chloroplast precursor	+	+	C	Y	4	0,461	22,91	10,28
At4g25650	Tic55-like (HP62, ACD1-Like) Rieske (2Fe-2S) protein domain-containing protein, chloroplast precursor	+	-	C	Y	2	-0,34	61,26	8,9
At2g34460	Ycf39-like (HP26c) (Tic62-like) NAD-dependent epimerase,dehydratase family protein	+	+	C	N	ND	-0,053	30,47	9,03
At3g46780	Ycf39-like (Tic62-like) NAD-dependent epimerase,dehydratase family protein	-	+	C	Y	ND	-0,349	54,35	8,9
At2g15290	Tic21 (Cia5) or PIC1 translocon at the inner envelope membrane or Iron transporter, chloroplast precursor	-	+	C	Y	4	0,315	31,27	10,23
At2g28900	OEP16-1 (HP15) mitochondrial import inner membrane translocase subunit Tim17/Tim22/Tim23 family protein	+	+	-	N	ND	0,12	15,48	9,16
At5g55510	OEP16-like (HP22) mitochondrial import inner membrane translocase subunit Tim17/Tim22/Tim23 family protein	+	-	-	N	ND	0,04	22,52	9,08
At3g49560	OEP16-like (HP30) mitochondrial import inner membrane translocase subunit Tim17/Tim22/Tim23 family protein, dual targeted ?	+	+	-	N	3	-0,124	27,98	9,49
At5g24650	OEP16-like (HP30-2) mitochondrial import inner membrane translocase subunit Tim17/Tim22/Tim23 family protein, dual targeted	+	+	-	N	3	-0,125	27,77	9,6
At4g26670	OEP16-like mitochondrial import inner membrane translocase subunit Tim17/Tim22/Tim23 family protein	+	-	-	N	2	0,136	21,81	7,62
At1g02280	Toc33 (HP32b) GTP-binding protein, chloroplast outer envelope translocon subunit (EC 3.6.5.-)	+	+	-	N	ND	-0,139	32,92	9,1
At5g05000	Toc34 (OEP34) GTP-binding protein, chloroplast outer envelope translocon subunit (EC 3.6.5.-)	+	+	-	N	ND	-0,151	34,7	9,42
At3g17970	Toc64-III (HP64b) chloroplast outer envelope translocon subunit	+	+	S	N	ND	-0,137	64,02	8,61
At3g46740	Toc75-III (OEP75-3) chloroplast outer envelope import-associated channel, chloroplast precursor	+	+	C	Y	ND	-0,365	89,19	8,93
At5g19620	Toc75-V (OEP85, OEP80, IAP75) chloroplast outer envelope import-associated channel, chloroplast precursor	-	+	C	Y	ND	-0,365	79,93	8,41
At4g02510	Toc159 (OEP86) chloroplast outer envelope protein import component	+	+	-	N	ND	-0,479	160,82	4,42
Redox									
At3g26060	PrxQ peroxiredoxin Q, chloroplast precursor	-	+	C	Y	ND	-0,402	23,67	9,53
At3g59840	P1-like NADP-dependent oxidoreductase P1-like protein (EC 1.3.1.74)	-	+	C	N	ND	-0,31	10,5	4,64
At4g13010	ceQORH (IE41) quinone oxidoreductase, uncleaved chloroplast precursor	+	-	-	N	ND	0,034	34,43	9,04

At1g06690	HP52 aldo/keto reductase family protein	+	+	C	Y	ND	-0,275	41,49	8,85
At2g27680	conserved protein of the aldo/keto reductase family	-	+	C	Y	ND	-0,292	43,15	8,2
At1g71500	Rieske (2Fe-2S) domain-containing protein	-	+	C	Y	ND	-0,226	31,72	8,88
At3g11630	PrxB (BAS1) 2-cys peroxiredoxin, chloroplast precursor	-	+	C	Y	ND	-0,112	29,09	6,91
At4g35460	TRXB1 (NTR1, NRTB) NADPH-dependent thioredoxin reductase 1 (EC 1.8.1.9)	-	+	C	N	ND	-0,038	39,62	6,95
RNA binding									
At2g43630	conserved glycine-rich plant protein	-	+	C	Y	1	-0,597	30,7	8,29
At5g22640	MORN repeat-containing, glutamic acid-rich protein (EMB1211) restricted to Arabidopsis, no similarity to other protein (even plant proteins)	-	+	-	N	ND	-0,882	99,95	4,36
At4g13130	CHP-rich zinc finger protein, DC1 domain-containing protein	-	+	-	N	ND	-0,341	89,62	6,02
At1g09340	Rap38 (CSP41B) putative RNA-binding protein	-	+	-	N	ND	-0,364	42,62	8,18
At3g63140	Rap41 (CSP41A) mRNA binding protein, chloroplast precursor	-	+	C	Y	ND	-0,17	43,93	8,54
At2g37220	ROC1 (CP29B) putative RNA-binding protein, chloroplast precursor	-	+	C	Y	ND	-0,323	30,71	5,05
At1g55480	TPR repeat containing protein, similarity to tyrosine phosphatase	-	+	C	Y	ND	-0,709	37,41	8,18
Signaling (other than lipid signaling)									
At3g51140	HP28 (AtCDF1-like) cell growth defect factor, domain HSP DnaJ, similar to Slr1918 protein [Synechocystis sp]	+	+	C	Y	4	-0,016	31,53	10,66
At5g23040	HP28-like (AtCDF1) cell growth defect factor, domain HSP DnaJ, similar to Slr1918 protein [Synechocystis sp]	-	+	C	Y	3	0,163	28,81	9,68
At5g64430	octicosapeptide/Phox/Bem1p (PB1) domain-containing protein	-	+	-	N	ND	-0,732	56,44	5,6
Stress (biotic & abiotic)									
At3g18890	UOS1-like similar to UV-B and ozone regulated protein 1	-	+	C	Y	ND	-0,37	68,34	8,27
At3g12570	SLT1-like protein, sodium-lithium tolerant protein 1, contains domain HSP20-like chaperone	-	+	-	N	ND	-0,537	55,27	6,04
At1g30360	ERD4 early-responsive to dehydration stress protein 4	-	+	S	N	10	0,298	81,93	9,28
At2g43940	TMT2-like similar to thiol methyltransferase 2	-	+	-	N	ND	-0,25	25,04	5,66
Stress (oxidative)									
At2g25080	GPX2 (GPX1, PHGPx) phospholipid hydroperoxide glutathione peroxidase 1, chloroplast precursor	+	+	C	Y	ND	-0,173	26,01	9,41
At1g11840	GLX1 glyoxalase I-2, lactoylglutathione lyase-like, putative	-	+	-	N	ND	-0,336	31,92	5,19
At4g35000	APX3 L-ascorbate peroxidase 3, dual targeting to chloroplast and mitochondria	+	+	-	N	1	-0,365	31,57	6,46
Translation (cytosolic)									
At1g06380	RSL1D1-like Ribosomal L1 domain-containing protein	-	+	C	-	ND	-0,255	28,71	9,48
Transporters									
At2g42770	HP25 PMP22-like peroxisomal membrane 22 kDa family protein	+	+	-	Y	2	-0,144	25,91	9,81
At5g19750	HP25-like (HP30c) PMP22-like peroxisomal membrane 22 kDa family protein	+	-	C	Y	3	0,202	30,36	10,23
At5g62720	IEP18 integral membrane HPP family protein, weak similarity to	+	+	C	Y	5	0,52	25,82	10,14

	various transporter families								
At5g52540	HP47 conserved membrane protein family, weak similarity to various transporter families	+	-	C	Y	9	0,757	47,64	9,96
At1g32080	HP45 LrgB-like family protein, conserved membrane protein	+	+	C	Y	12	0,701	54,01	9,63
At3g60590	HP36b weak similarity to branched-chain amino acid transport system II carrier protein	+	-	M	Y	5	0,654	26,27	9,6
At5g58270	ABC (STA1, STARIK 1) mitochondrial half-ABC transporter	-	+	M	Y	6	-0,017	80,42	9,27
At5g03910	ABC (ATATH12) ABC-2 homologe 12, similar to sll1276 protein [Synechocystis sp]	-	+	C	Y	6	0,23	69,19	9,05
At4g25450	NAP8 (HP77) Non-intrinsic ABC protein 8, chloroplast precursor	+	+	M	Y	5	0,205	77,92	9,12
At4g33460	NAP13 (EMB2751) Non-intrinsic ABC protein 13, chloroplast precursor	-	+	C	Y	ND	-0,046	29,62	8,68
At1g80300	TLC1 (AATP1, AtNTT1) chloroplast ADP, ATP carrier protein 1, chloroplast precursor	-	+	C	Y	11	0,368	68,13	9,39
At1g15500	TLC2 (AATP2, AtNTT2) chloroplast ADP,ATP carrier protein 2, chloroplast precursor	+	-	M	Y	11	0,412	67,53	9,51
At4g37270	HMA1 (AHM1) metal-transporting P-type ATPase, chloroplast precursor	+	-	M	Y	6	0,119	88,19	8,05
At5g59250	HP59 sugar transporter family	+	+	C	Y	11	0,549	59,83	9,02
At5g16150	IEP62 (GLT1 /PGLCT) putative glucose transporter, chloroplast precursor	+	+	C	Y	12	0,592	56,97	9,06
At4g00290	MscS (MsL) mechanosensitive ion channel domain-containing protein	-	+	M	Y	5	0,092	53,88	9,06
At1g48460	weak similarity to MscS Mechanosensitive ion channel	-	+	C	Y	5	0,313	38,07	9,68
At5g43745	HP88-like Ion channel DMI1-like, castor/pollux family	-	+	C	Y	2	-0,086	92,21	9,14
At5g02940	HP88 ion channel DMI1-like, castor/pollux family	+	+	M	N	3	-0,172	92,13	7,53
At1g01790	KEA1 (HP64) K+ efflux antiporter, putative	+	+	-	N	13	0,665	64,98	6,1
At4g00630	KEA2 (HP64-like) K+ efflux antiporter, putative	-	+	M	N	12	0,628	66,41	7,07
At3g20320	TGD2 (HP41b) phosphatidic acid-binding protein, chloroplast precursor	+	+	C	Y	1	-0,04	41,63	8,9
At5g17520	MEX1 (RCP1) maltose transporter (root cap 1), chloroplast precursor	+	-	C	Y	9	0,409	45,28	9,37
At5g01500	MCF (HP45b) mitochondrial substrate carrier family protein	+	-	C	Y	3	-0,079	45,09	9,81
At2g35800	MCF (HP90) mitochondrial substrate carrier family protein	+	-	-	N	2	-0,125	90,62	8,95
At3g51870	MCF (HP42) mitochondrial substrate carrier family protein, similar to peroxisomal Ca-dependent solute carrier	+	-	C	Y	2	0,002	41,81	9,75
At5g22830	AtMRS2-11 (GMN10) magnesium transporter 10, CorA-like family protein	-	+	C	Y	2	-0,182	51,09	5,23
At2g26900	IEP36 bile acid:sodium symporter family protein	+	-	C	N	9	0,641	43,6	8,95
At3g56160	IEP36-like weak similarity to bile acid:sodium symporter family	-	+	C	Y	7	0,57	46,53	9,91
At5g64290	DiT2-1 glutamate/malate translocator, chloroplast precursor	+	+	C	Y	10	0,607	59,99	9,33
At5g12860	DiT1 (IEP45) 2-oxoglutarate/malate translocator, chloroplast precursor	+	+	C	Y	14	0,775	59,21	9,75

At3g26570	PHt2-1 (IEP60) phosphate transporter, chloroplast precursor	+	+	-	N	12	0,47	64,78	9,26
At5g33320	PPT (IEP33, CUE1) phosphate/phosphoenolpyruvate translocator, chloroplast precursor	+	-	C	Y	6	0,434	44,22	10,16
At5g46110	TPT (IEP30, APE2) phosphate/triose-phosphate translocator, chloroplast precursor	+	+	M	Y	8	0,549	44,63	9,75
At4g39460	MCF SAMC1/SAMT1 (HP35) S-Adenosylmethionine transporter, dual targeted (chloroplast, mitochondria)	+	-	C	Y	5	0,1	34,86	9,68
At1g76405	OEP21-1 ATP-regulated anion-selective solute channel	+	-	-	N	ND	-0,769	19,45	9,49
At3g52230	OEP24 (OMP24) chloroplast outer envelope high-conductance solute channel	+	+	-	N	ND	-0,99	16,12	4,51
At5g42960	OEP24-II chloroplast outer envelope high-conductance solute channel	-	+	-	N	ND	-0,271	23,41	9,26
At2g43950	OEP37 (HP44) chloroplast outer envelope ion channel	+	+	C	Y	ND	-0,538	38,83	9,16
Vesicle formation & trafficking									
At2g20890	THF1 (TF1, PSB29) thylakoid formation1 protein, chloroplast precursor	-	+	C	Y	ND	-0,387	33,79	9,2
At1g65260	Vipp1 (IM30, PspA, HCF155, PTAC4) vesicle-inducing protein in plastids 1	+	+	C	Y	ND	-0,561	36,39	9,17
At5g17670	PGAP1-like conserved plant and cyanobacteria protein, lipase protein family	-	+	M	Y	ND	-0,131	33,4	5,57
At2g40060	weak similarity to Clathrin subfamily proteins	-	+	-	N	ND	-0,916	28,83	4,9
At3g22520	CAP1-like protein, CDPK adapter-like protein	-	+	-	N	ND	-0,811	67,57	5,29
Unknown									
At5g03900	conserved plant and cyanobacterial protein, similar to Slr1603 protein [Synechocystis sp], contains HesB/YadR/YfhF (Iron sulfur assembly protein IscA) domain	-	+	C	Y	ND	-0,34	59,5	9,19
At4g31530	conserved plant and cyanobacterial protein, similar to Sll0096 protein [Synechocystis sp.], contains NAD-dependent epimerase/dehydratase domain	-	+	C	Y	ND	-0,173	35,22	7,67
At3g11560	conserved plant protein, contains InterPro and PFAM domain LETM1-like protein	-	+	C	Y	ND	-0,373	97,79	6,46
At2g36835	conserved plant protein, no similarity to characterized protein	-	+	C	N	ND	-0,053	13,38	7,84
At1g42960	HP17 conserved protein, similar to Ssl2009 protein [Synechocystis sp], no similarity to characterized protein	+	+	C	Y	1	-0,188	17,82	8,54
At5g16660	HP17-like conserved protein, similar to Ssl2009 protein [Synechocystis sp], no similarity to characterized protein	-	+	C	Y	1	-0,479	18,17	8,51
At1g67080	HP23 conserved protein, similar to Sll0354 protein [Synechocystis sp]	+	-	C	N	4	0,316	24,62	9,44
At4g27990	HP24 Ycf19-like YGGT family protein, conserved plant and cyanobacterial protein (chloroplast encoded in algae), similarity to ssr2142 protein [Synechocystis sp]	+	-	C	Y	3	0,393	23,7	11,28
At3g07430	HP24-like Ycf19-like YGGT family protein, conserved plant and cyanobacterial protein, similar to Ycf19 protein [Synechocystis	-	+	C	Y	ND	0,362	24,92	10,66

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At3g57280	HP26b conserved 14c-like transmembrane protein	+	+	C	Y	4	0,108	24,34	9,17
At2g38550	HP26b-like (HP36c) conserved 14c-like transmembrane protein	+	+	C	Y	4	-0,155	36,7	6,99
At3g43520	HP26b-like conserved 14c-like transmembrane protein	-	+	C	Y	4	0,093	24,76	9,13
At3g32930	HP27b conserved plant protein, no similarity to characterized protein	+	-	C	Y	ND	-0,361	27,43	9,6
At4g13590	HP28b conserved protein and cyanobacteria protein, similar to sll0615 protein [Synechocystis sp], no similarity to characterized protein	+	-	C	Y	6	0,439	37,93	8,82
At3g61870	HP29b conserved plant and cyanobacteria protein, similar to Slr1676 protein [Synechocystis sp], no similarity to characterized protein	+	+	C	Y	4	0,093	29,58	9,34
At5g13720	HP29c conserved plant protein, contains InterPro domain IPR000218 Ribosomal protein L14b/L23e	+	-	C	Y	3	0,324	28,91	9,02
At1g78620	HP34 conserved plant and cyanobacteria membrane protein, similar to protein sll0875 [Synechocystis sp]	+	-	C	Y	6	0,414	34,87	9,83
At3g08640	HP35b (LCD1-like) conserved plant protein, no similarity to characterized protein	+	-	C	Y	3	0,099	35,25	9
At5g12470	HP35b-like (HP40, LCD1-like) conserved plant protein, no similarity to characterized protein	+	+	C	Y	4	0,103	41,32	7,01
At5g22790	HP35b-like (HP46, LCD1-like) conserved plant protein, no similarity to characterized protein	+	-	C	Y	3	-0,127	46,83	5,14
At5g24690	HP35b-like (HP56b, LCD1-like) conserved plant protein, no similarity to characterized protein	+	+	C	Y	3	-0,133	56,81	9,14
At1g20830	HP40b conserved plant protein, no similarity to characterized protein	+	-	C	Y	1	-0,622	39,41	9,09
At2g44640	HP50 (PDE320-like) conserved plant protein, no similarity to characterized protein	+	+	C	N	ND	-0,236	49,83	8,8
At5g08540	HP53 conserved plant protein, no similarity to characterized protein	+	+	C	N	1	-0,404	38,65	5,67
At5g01590	HP65 expressed protein, no similarity to characterized protein	+	+	C	Y	ND	-0,731	48,87	6,38
At5g23890	HP103 conserved protein, S-layer domain-containing protein, similar to slr2000 protein [Synechocystis sp]	+	+	C	Y	1	-0,518	103,92	4,61
At4g13670	PTAC5 DnaJ domain-containing protein, similar to Alr2745 protein [Anabaena sp]	-	+	C	Y	ND	-0,595	44,02	4,84
At1g16790	conserved plant protein, no similarity to characterized protein	-	+	-	N	ND	-0,41	15,45	9,55
At3g18420	HP35c tetratricopeptide repeat (TPR) containing protein	+	-	C	Y	ND	-0,325	35,63	5,24
At2g37400	TPR repeat containing protein, weak similarity with the Slr1644 protein [Synechocystis sp]	-	+	C	Y	ND	-0,463	38,15	6,7
At2g01220	conserved protein, no similarity to characterized protein	-	+	-	Y	ND	-0,037	42,14	6,27
At3g63160	OEP6 chloroplast outer envelope membrane protein, no similarity to characterized protein	+	+	-	N	1	-0,157	7,25	9,04
At2g34585	OM14 similar to Outer envelope membrane protein [Pisum	+	-	S	-	ND	-0,13	8,57	4,03

	sativum]								
At2g11910	aspolin1-like protein, aspartic acid-rich protein,	-	+	-	N	1	-1,407	18,49	3,59
At1g19100	conserved eukaryote protein, ATPase-like domain	-	+	-	N	ND	-0,426	74,17	7,7
At3g22620	conserved plant protein, weak similarity with non-specific lipid-transfer protein (LTP) family protein	+	-	S	Y	2	0,151	20,77	6,09
At1g67700	conserved plant protein, no similarity to characterized protein, oligopeptidase/protease domain	-	+	M	N	ND	-0,475	26,01	9,57
At2g31190	HP48 (EMB1879-like) conserved plant protein, no similarity to characterized protein	+	-	-	N	2	-0,081	48,25	7,7
At1g13930	conserved plant protein, no similarity to characterized protein	-	+	-	N	ND	-0,883	16,16	4,82
At4g00640	HP57 conserved plant protein, weak similarity with protein sll1021 [Synechocystis sp], no similarity to characterized protein	+	-	-	N	1	-0,523	51,41	4,82
At3g28220	conserved plant protein with PPR signature	-	+	-	-	1	-0,425	42,88	8,59
At2g37930	expressed protein, no similarity to characterized protein, Transcriptional factor tubby domain	-	+	-	N	ND	-0,665	51,96	8,83
At2g47750	GH39 auxin-responsive GH3 family protein	-	+	-	N	ND	-0,11	66,15	6,08
At2g40550	conserved protein, restricted to eukaryotes	-	+	-	N	ND	-0,267	65,75	5,18
At5g51320	protein restricted to Arabidopsis, no similarity to any other protein (even plant proteins)	-	+	-	N	ND	0,101	13,1	10,12