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The Distinct Functional Roles of the Inner and Outer Chloroplast Envelope of Pea (*Pisum sativum*) As Revealed by Proteomic Approaches

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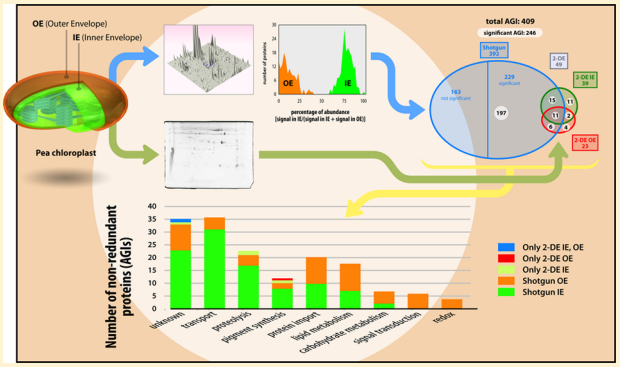
Supporting Information

ABSTRACT: Protein profiles of inner (IE) and outer (OE) chloroplast envelope membrane preparations from pea were studied using shotgun nLC−MS/MS and two-dimensional electrophoresis, and 589 protein species (NCBI entries) were identified. The relative enrichment of each protein in the IE/OE pair of membranes was used to provide an integrated picture of the chloroplast envelope. From the 546 proteins identified with shotgun, 321 showed a significant differential distribution, with 180 being enriched in IE and 141 in OE. To avoid redundancy and facilitate *in silico* localization, *Arabidopsis* homologues were used to obtain a nonredundant list of 409 envelope proteins, with many showing significant OE or IE enrichment. Functional classification reveals that IE is a selective barrier for transport of many metabolites and plays a major role in controlling protein homeostasis, whereas proteins in OE are more heterogeneous and participate in a wide range of processes. Data support that metabolic processes previously described to occur in the envelope such as chlorophyll and tocopherol biosynthesis can be ascribed to the IE, whereas others such as carotenoid or lipid biosynthesis occur in both membranes. Furthermore, results allow empirical assignation to the IE and/or OE of many proteins previously assigned to the bulk chloroplast envelope proteome.



KEYWORDS: *2-DE electrophoresis, chloroplast envelope, database, membrane proteins, pea, Pisum sativum, shotgun proteomics*

INTRODUCTION

Chloroplasts, the green photosynthetic plastids responsible for energy capture in plants, are surrounded by a double membrane system, the chloroplast envelope. The chloroplast envelope consists of an inner (IE) and an outer (OE) membrane and controls the bidirectional traffic of a diverse range of substances between the cytosol and the biochemically active chloroplast interior and therefore is essential for proper cell functioning. Because the chloroplast is a semiautonomous organelle, nuclear encoded proteins need to be transported across this membrane system and this process is achieved via the joint action of translocons located at the OE and IE (TOC and TIC, respectively; reviewed in refs 1−3). In addition, the envelope controls the exchange of many ions and metabolites between the chloroplast stroma and the cytosol. For instance, inorganic nitrite and sulfate need to cross the envelope to be reduced within the chloroplast, whereas amino acids and fatty acids synthesized in the stroma have to be exported for their distribution within the cell. Further, secondary metabolites such as cofactors and vitamins are also likely to be transported across the chloroplast envelope. Accordingly, metabolite and ion transporters have been found in OE and IE membrane preparations from several plant species (for overview see refs 4−8).

The envelope is not only a selective barrier between the chloroplast and the cytosol, since several unique biosynthetic pathways are present in this membrane system and the OE is

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also implicated in organelle movement (reviewed in refs 9−11). The chloroplast envelope plays an important role in certain aspects of fatty acid and glycerolipid metabolism, including biosynthesis and desaturation, and also contains enzymes involved in the formation of isoprenoid plastid constituents such as carotenoids, chlorophylls, and prenyl-quinones.[11] Envelopes are also involved in signaling, since they are the site for production of molecules such as jasmonate and abscisic acid, which are involved in plastid-to-nucleus communication.[11] On the other hand, the presence in the envelope of antioxidant enzymes such as ascorbate and gluthatione peroxidases has led to the hypothesis that the envelope participates in redox defense mechanisms.[11,12]

A substantial body of knowledge on envelope proteins has arisen from proteomic approaches using spinach and *Arabidopsis thaliana*.[13−15] In spinach, a proteomic approach identified 54 proteins, 27 of them not previously reported in this plant compartment.[14] Subsequently, a combined proteomic and *in silico* study reported more than 100 envelope proteins in *Arabidopsis*.[13] A curated list, including 226 envelope proteins, was later published using a combination of the data reported until then,[11] and finally AT_Chloro, a database dedicated to subplastidial localization of *Arabidopsis* chloroplast proteins, was released.[16,17] A total of 1323 proteins were identified in chloroplasts, and statistical analysis allowed the accurate localization of 819 proteins after cross-contamination analyses. From this subset, "the whole envelope proteome" included 460 proteins, whereas "the specific envelope proteome" (that containing proteins with increased abundance in the envelope when compared to other subplastidial compartments) included 298 proteins. Their functional classification indicated that unknown, metabolism, transport, translation, chaperone, and protease protein classes comprised more than 80% of the chloroplast envelope proteins.[16,17] Recently, the chloroplast envelope proteomes of *Pisum sativum*, *A. thaliana*, and *Medicago sativa* were compared, and 247 envelope proteins were identified.[18] In addition, comparative proteomics between pea and maize has shown that C_3- and C_4-type envelopes have qualitatively similar but quantitatively different membrane proteins.[19]

The allocation of *A. thaliana* chloroplast envelope proteins to the OE and IE has been mainly performed based on *in silico* studies and extensive literature search.[16,17] This approach has some limitations, especially for the identification of OE proteins, most of which are synthesized without a classical N-terminal chloroplast targeting sequence. Recently, the proteome of OE and IE from pea has been studied using nHPLC-MALDI/Q-TOF, and 49 and 50 proteins were assigned to the IE and OE, respectively.[18] Overall, the proteomic characterization of the IE and OE membranes is challenging due to (i) the difficulties in obtaining highly purified IE and OE membrane fractions, (ii) the inherent problems involving the separation and solubilization of highly hydrophobic proteins within these fractions, and (iii) the relative low abundance of the envelope proteome, which accounts for only 1−2% of the chloroplast proteins.[17] A deeper knowledge of the protein profile of these membrane subsystems will thus help clarify their respective roles in chloroplast functioning and biogenesis.

The aim of the present study was to gain further knowledge on the specific protein profiles of the IE and OE membranes from the chloroplast envelope of *P. sativum* (pea), using two complementary proteomic approaches, 2-DE and label-free shotgun proteomics. 2-DE electrophoresis has been widely used in plant proteomics,[20] and it is still a powerful approach to separate complex protein mixtures. However, this technique has limitations that make difficult to detect certain groups of proteins, such as those with low abundance or highly hydrophobic. Therefore, a more sensitive and complementary approach, label-free shotgun proteomics, was also pursued. This latter approach has been successfully used to study the proteome of other plant membranes such as the plasma membrane.[21,22] Pea was chosen as a model system because the method for the separation of IE and OE membranes is well established in these species[23] and available EST sequencing data[24] facilitates annotation and comparison with *A. thaliana* genes.

EXPERIMENTAL PROCEDURES

Plant Material and Envelope Membrane Preparation

Pea plants (*P. sativum* L., cv. "Arvica", Prague, Czech Republic) were grown on sand in the greenhouse under a 16 h light (220 μmol m^{-2} s^{-1})/8 h dark regime at 21 °C. Preparations of OE and IE membrane fractions were obtained from intact, Percoll-purified chloroplasts, isolated from leaves of 10-day-old pea plantlets as described elsewhere.[23,25] Chloroplasts were ruptured in a Dounce-homogenizer after hypertonic treatment in 0.65 M sucrose on ice, and readjusted to isotonic conditions by diluting 2-fold. Intact chloroplasts and thylakoid membranes were removed by low speed centrifugation (250 mL beakers in a fixed-angle JA14 rotor, at 4150*g*, 4 °C, 10 min), followed by a second 2-fold dilution and centrifugation at 196000*g*, 4 °C for 1 h. The light-green supernatant consisting of crude envelope membranes was removed, and IE and OE membranes were separated on a discontinuous sucrose gradient (0.996, 0.800, and 0.465 M) by centrifugation (137000*g*, 4 °C, 3 h), resulting in an opaque (OE) and a light yellow-green (IE) fraction. Each fraction was diluted 4-fold, and after pelleting at 137000*g*, 4 °C, 1 h, membranes were resuspended in 0.5 M Na_3PO_4 buffer (pH 7.9) and frozen in liquid N_2. Final protein concentration was approximately 2.5 μg μL^{-1}. Experimental details and buffer compositions during isolation were as in ref 23. In order to obtain a broad coverage of envelope proteins, including integral membrane proteins as well as those only associated with the membrane, we did not carry out further purification steps. One-dimensional gels of representative IE and OE fractions are shown in Figure S1 of the Supporting Information (SI).

Protein concentration in the samples was measured with the Bradford method, using microtiter plates, an Asys UVM 340 (Biochrom Ltd., Cambridge, U.K.) spectrophotometer, and BSA as standard.

Label Free Nanoliquid Chromatography-Tandem Mass Spectrometry (nLC−ESI−MS/MS)

Label-free nLC−ESI−MS/MS analysis was made with four independent biological replicates (i.e., each one from a different batch of plants) for the two membrane types (IE and OE). Sample preparation for label-free nLC−ESI−MS/MS was carried out according to ref 21. Briefly, purified OE and IE fractions were subjected to 1-DE to remove nonprotein compounds. The resulting gel bands were cut into four pieces, proteins were *in gel* digested with trypsin, and subsequently, peptides were extracted.

Peptide solutions were concentrated in a trap column (Lcolumn Micro 0.3 mm × 5 mm; CERI, Japan) using an ADVANCE UHPLC system (Michrom Bioresources, Auburn, CA, U.S.A.). Elution was carried out with 0.1% (v/v) formic

acid in ACN, and concentrated peptides were separated in a Magic C_{18} AQ nano column (0.1 mm × 150 mm; Michrom Bioresources) using a linear gradient of ACN (from 5% to 45%) and a flow rate of 500 nL min^{-1}. Ionization of peptides was carried out with a spray voltage of 1.8 kV using an ADVANCE spray source (Michrom Bioresources). Mass spectrometry analysis was carried out on an LTQ Orbitrap XL (Thermo Fisher Scientific, Waltham, MA, U.S.A.) equipped with Xcalibur software (version 2.0.7, Thermo Fisher Scientific). Normalized collision energy and isolation width were set to 35% and 2 m/z, respectively. Dynamic exclusion was enabled with the following parameters: repeat count, 1; repeat duration, 30 s; exclusion list size, 500; exclusion duration, 60 s; and exclusion mass width, ± 0.01 m/z. Under the data-dependent scanning mode, full-scan mass spectra were obtained in the range of 400−1800 m/z with a resolution of 30000. Collision-induced fragmentation was applied to the five most intense ions at a threshold above 500.

Mass data analysis was performed according to ref 22. For semiquantitative analysis of IE and OE membrane proteins, experimental raw MS/MS data files were analyzed with Progenesis LC−MS software (version 4.0, Nonlinear Dynamics, Newcastle, U.K.) according to the software's instructions, and a list of peptides was obtained. In this process a reference run was selected, and retention times of each run were aligned. Each feature was normalized based on the quantitative abundance ratio. Peptide abundances were compared using ANOVA ($p < 0.05$). Protein identification was carried out using the full peptide list with the Mascot search engine (version 2.3.02, Matrix Science, London, U.K.) and the NCBInr Green Plants database (version 20120616; 18538238 sequences; 6354146082 residues). Search parameters were: peptide mass tolerance ±5 ppm, MS/MS tolerance ±0.6 Da, one allowed missed cleavage, allowed fixed modification carbamidomethylation (Cys), and variable modification oxidation (Met) and peptide charges were set to +1, +2 and +3. Positive protein identification was assigned with at least two unique top-ranking peptides with scores above the threshold level ($p < 0.05$). Protein information was exported from Mascot xml format and imported to Progenesis software, which then associates peptide and protein information. Full information about peptides identified and quantified by shotgun proteomic and Progenesis LC−MS analyses is listed in the Table S1 of the SI. Only proteins identified in all four biological replicates of each sample type were considered for the analysis.

2-DE

Gels were made from two independent IE and OE membrane preparations (i.e., each from a different batch of plants) with four technical replicates each ($n = 8$). Preliminary 2-DE experiments were carried out using a first-dimension IEF separation with a linear pH gradient 3−10. In these conditions, most of the spots were located in the central region of the 2-DE gel in the OE preparations and evenly distributed throughout the gel for the IE preparations (results not shown); therefore, to prevent protein comigration and improve resolution, a narrower pH gradient (5−8) was chosen for the OE samples. First-dimension IEF separations were carried out in a Protean IEF Cell (BioRad, Hercules CA, U.S.A.) on 7 cm ReadyStrip IPG Strips (BioRad) with linear pH gradients 5−8 for OE and 3−10 for IE preparations. Strips were passively rehydrated for 16 h at 20 °C in 125 μL of rehydration buffer containing 80 μg (for OE) or 45 μg (for IE) of envelope proteins and a trace of bromophenol blue, and then transferred onto a strip electrophoresis tray. IEF was run at 20 °C, for a total of 14000 V h (20 min with a 0−250 V linear gradient, 2 h with a 250−4000 V linear gradient, and 4000 V until 10000 V h). After IEF, strips were equilibrated for 10 min in equilibration solution I [6 M urea, 0.375 M Tris-HCl, pH 8.8, 2% (w/v) SDS, 20% (v/v) glycerol, 2% (w/v) DTT] and for another 10 min in equilibration solution II [6 M urea, 0.375 M Tris-HCl pH 8.8, 2% (w/v) SDS, 20% (v/v) glycerol, 2.5% (w/v) iodoacetamide].

For the second-dimension SDS-PAGE, equilibrated IPG strips were placed on top of vertical 12% SDS-polyacrylamide gels (8 cm × 10 cm × 0.1 cm) and sealed with melted 0.5% agarose in 50 mM Tris-HCl, pH 6.8, containing 0.1% SDS. SDS-PAGE was carried out at 20 mA per gel for approximately 1.5 h, until the bromophenol blue reached the plate bottom, in a buffer containing 25 mM Tris, 192 mM glycine, and 0.1% SDS, at 4 °C. Gels were subsequently stained with Coomassie-blue G-250 (Serva, Barcelona, Spain).

Gel Image and Statistical Analysis. Stained gels were scanned with an Epson Perfection 4990 Photo scanner at 600 dpi (Epson Corporation, Barcelona, Spain). Experimental M_r values were calculated by mobility comparisons with Precision Plus protein standard (BioRad) run in a separate lane on the SDS-gel, and pI was determined by using a 3−10 linear scale for IE and 5−8 for OE preparations. Spot detection, gel matching and interclass analysis were performed with PDQuest 8.0 software (BioRad). First, normalized spot volumes based on total intensity of valid spots were calculated for each 2-DE gel and used for statistical calculations of protein abundance; for all spots present in the gels, pI, M_r, and normalized volumes (mean values, SD, and CV) were determined. Only spots consistently present in 100% of the replicates (all eight gels) were considered as consistent and used in further analysis. The spots were also manually checked, and a consistent reproducibility between normalized spot volumes was found in the different replicates.

Protein *in Gel* Digestion of 2-DE Spots. Spots were excised automatically using a spot cutter EXQuest (BioRad), transferred to 500 μL Protein LoBind Eppendorf tubes, destained in 400 μL of 40% [v/v] acetonitrile (ACN) and 60% [v/v] 200 mM NH_4HCO_3 for 30 min and dehydrated in 100% ACN for 10 min. Gel pieces were dried at room temperature and then *in gel* digested with 15 μL Trypsin solution (Sequencing grade Modified Trypsin V511, Promega, Madison, WI, U.S.A.; 0.1 μg μL^{-1} in 40 mM NH_4HCO_3/9% ACN). After incubation for 5 h at 37 °C, the reaction was stopped by adding 1 μL of 1% TFA. The peptide solution was finally analyzed using mass spectrometry (MS).

Protein Identification by Nanoliquid Chromatography−Tandem Mass Spectrometry (nLC−ESI−MS/MS) of 2-DE Spots. Peptides present in 5 μL of sample were preconcentrated *on line* onto a 300 μm i.d. × 5 mm, 5 μm particle size ZORBAX 300SB-C18 trap column (Agilent Technologies, Waldbronn, Germany), using a 100 μL min^{-1} flow rate of 3% ACN, 0.1% formic acid, in a nano-HPLC system 1200 series (Agilent Technologies). Backflow elution of peptides from the trap column was carried out and separation done with a 75 μm i.d. × 150 mm, 3.5 μm particle size ZORBAX 300SB-C_{18} column (Agilent Technologies), using a 300 nL min^{-1} nanoflow rate and a 55 min linear gradient from solution 97% A (0.1% formic acid) to 90% of solution B (90% ACN, 0.1% formic acid). The nano-HPLC was connected to an

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HCT Ultra high-capacity ion trap (Bruker Daltoniks, Bremen, Germany) using a PicoTip emitter (50 μm i.d., 8 μm tip i.d., New Objective, Woburn, MA, U.S.A.) and an on line nanoelectrospray source. Capillary voltage was −1.8 kV in positive mode, and a dry gas flow rate of 10 L min^{-1} was used with a temperature of 180 °C. The scan range used was from 300 to 1500 m/z. The mass window for precursor ion selection was ±0.2 Da, and the rest of parameters were those recommended by the manufacturer for MS/MS proteomics work. Peak detection, deconvolution, and processing were performed with Data Analysis 3.4 (Bruker Daltonics).

Protein identification was carried out using the Mascot search engine (Matrix Science) and the nonredundant databases NCBInr 20120204 (17172511 sequences; 5891506632 residues) and Plants_EST EST_110 (151512996 sequences; 26700120026 residues). Search parameters were monoisotopic mass accuracy, peptide mass tolerance ±0.2 Da, fragment mass tolerance ±0.6 Da, one allowed missed cleavage, allowed fixed modification carbamidomethylation (Cys), and variable modification oxidation (Met). Positive identification was assigned with Mascot scores above the threshold level ($p < 0.05$), at least two identified peptides with a score above homology, 10% sequence coverage, and similar experimental and theoretical molecular weight and pI values. The list of peptides identified is shown in Table S2 of the SI. We used the GO biological process annotation (http://www.geneontology.org) of the individual identified proteins for classification.

Protein Identification by MALDI-TOF MS analysis. For MALDI-TOF MS, 0.5 μL of the matrix solution (5 mg α-cyano-4-hydroxycinnamic-acid in 50% v/v ACN and 0.25% w/v TFA) were placed onto the MALDI target and dried at RT. Then, 2 μL of the digested sample was applied on top, allowed to dry, and topped with 0.5 μL of the matrix solution. Tryptic peptides were analyzed with a SHIMADZU AXIMA-CFR mass spectrometer (Shimadzu Scientific Instruments, Columbia, U.S.A.). Spectra acquisitions were done using AXIMA software (Shimadzu).

Protein identification was performed by searching in the nonredundant NCBInr 20120226 (17406376 sequences; 5965441494 residues), and Plants_EST EST_110 (151512996 sequences; 26700120026 residues) databases, using the Mascot search engine (Matrix Science, London, UK) and the following parameters: monoisotopic mass accuracy, fragment mass tolerance ± 0.6 Da; missed cleavages, 0 and 1; allowed variable modifications, oxidation (Met) and carbamidomethylation (Cys). Positive identification was retained with MASCOT scores above the threshold level ($p < 0.05$), at least five peptides matched, sequence coverage above 20% and similar experimental and theoretical molecular weight and pI.

In Silico Analysis for Subplastidial Localization of Identified Pea Proteins

The UniProt entry and the GO biological process annotation were obtained from the NCBI entries using the PIR i.d. Mapping tool (pir.georgetown.edu/pirwww/search/idmapping.shtml). To minimize redundancy and facilitate the comparison with localizations predicted in the *Arabidopsis* At_-CHLORO,[16,17] SUBA3,[26] and Plant Proteome (PPDB)[27] databases, each protein species was assigned to the corresponding *Arabidopsis* ortholog (AGI nonredundant codes). Predictions of localization and presence of membrane-spanning domains as well as additional references for the proteins were acquired from the plant membrane protein database ARAMEMNON (http://aramemnon.botanik.uni-koeln.de/).[28]

Immunoblot Analysis. Equal amounts of protein from IE and OE membrane preparations were separated by SDS-PAGE and subjected to immunoblot analysis using antibodies directed against Ps-TIC110, Ps-TOC75-III, Ps-OEP37, At-PIC1, Ps-OEP16.1, At-LHCb1 and Ps-LSU. Except for At-LHCb1 antiserum, which was purchased from Agrisera (Vännäs, Sweden), all polyclonal antisera used were raised in rabbit (Pineda Antibody Service, Berlin, Germany). Antisera were used in 1:500 to 1:5000 dilutions in TTBS buffer [100 mM TRIS-HCl, pH 7.5, 150 mM NaCl; 0.2% Tween-20; 0.1% bovine serum albumin (BSA)]. Nonspecific signals were blocked by 3% skim milk powder and 0.03% BSA. Secondary antirabbit IgG alkaline phosphatase (Sigma-Aldrich) was diluted 1:30000. Blots were stained for alkaline phosphatase reaction with 0.3 mg mL^{-1} nitroblue tetrazolium (NBT) and 0.16 mg mL^{-1} BCIP in 100 mM TRIS pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$. Signals were quantified by ImageQuant software (GE Healthcare).

RESULTS AND DISCUSSION

Protein Profiling of Membrane Preparations Enriched in IE and OE Using Shotgun Proteomics and 2-DE

The protein profiles of IE and OE membrane preparations from *P. sativum* (pea) were studied using a shotgun nLC−MS/MS approach. Four biological replicates were analyzed for each membrane type. A total of 546 protein species (NCBI entries) occurred in all biological replicates of at least one membrane type and were positively identified. Due to the inherent difficulties for obtaining highly pure IE and OE preparations and the high sensitivity of the methodology used, all but one of the proteins identified (545) were detected both in the IE and OE, although the relative abundances in each membrane fraction were very different in most of the cases. To determine the relative enrichment of proteins in the IE and OE fractions, a semiquantitative analysis was carried out using peptide signal intensity data. The relative percentage of abundance was estimated as signal in IE/(signal in IE + signal in OE). From the 546 identified protein species, 321 showed significant differences in relative abundance between the IE and OE preparations (ANOVA, $p < 0.05$; Table S3 of the SI). Two groups of proteins were clearly separated, with 180 and 141 of them having larger signal intensities in IE and OE preparations, respectively (Figure 1). The 225 proteins not showing significant differences in signal intensity between the IE and OE preparations are listed in the Table S4 of the SI. Localization and functional assignment was only assessed in proteins showing significant differences in relative abundance between IE and OE preparations (Table S3 of the SI). As a verification strategy for this semiquantitative approach, seven proteins were selected for immunoblot analysis including five that were identified and quantified with more than one peptide (Protein numbers 51, 66, 114, 129, and 132 in Table S3 of the SI) and two that were quantified with only one peptide (Protein numbers 154 and 177 in Table S3 of the SI). The latter two proteins represent contamination from stroma (LSU) and thylakoid membranes (LHC), respectively. Results from the immunoblots broadly confirm those found by the shotgun approach (Figure S2 of the SI).

The protein profiles of membrane preparations enriched in pea chloroplast IE and OE were also studied using 2-DE (IEF-

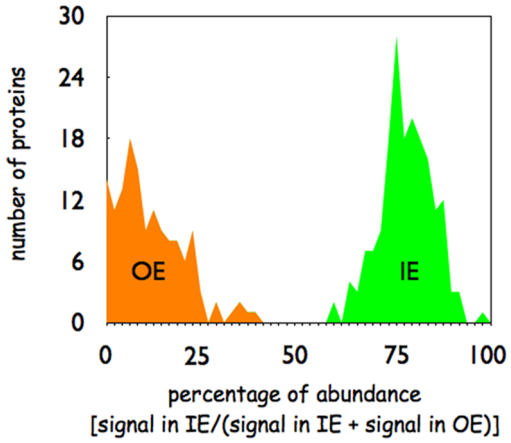

Figure 1. Allocation of protein species (NCBI entries) between OE and IE using shotgun proteomics. The relative percentage of abundance was estimated as signal in IE/(signal in IE + signal in OE). Only protein species showing significant differences in allocation between the IE (180 proteins) and OE (141 proteins) membrane preparations (ANOVA, $p < 0.05$) are shown.

SDS-PAGE electrophoresis). Two biological and four technical replicates were analyzed for each membrane type. Typical scans of 2-DE gels obtained from IE and OE membrane protein extracts are shown in Figure 2A,B, respectively. The average numbers of detected spots (mean ± SD; $n = 8$) in gels obtained from IE and OE membranes were 182 ± 8 and 189 ± 6, respectively. The numbers of consistent spots (those present in 100% of the gels of each membrane type) were 160 and 181 for the IE and OE preparations, respectively. All consistent spots were analyzed by nLC−MS/MS, and 92 and 59 spots in the IE and OE membrane preparations were unambiguously identified as protein species. The location of these spots is shown in the composite averaged maps in Figure S3 of the SI, and details on the protein identifications for each spot in the gels are shown in Tables S2 and S5 of the SI. These proteins corresponded to 50 and 33 NCBI protein species in IE and OE, respectively. The total number of NCBI protein species identified with the 2-DE approach was 69, because 14 of the proteins were found in both IE and OE.

In summary, a total of 589 protein species (NCBI entries) were identified in chloroplast envelopes using the two different proteomic approaches (Tables S3−S5 of the SI). The overlaps between the two techniques are shown using Venn diagrams (Figure 3A). From the 546 proteins identified with the shotgun approach, 321 (59%) show a significant differential distribution to IE or OE, and from these 295 (92%) were found only with this technique, whereas 26 (8%) were also found in the 2-DE gels (14 in IE, 5 in OE and 7 more both in IE and OE). Regarding the 2-DE approach, 69 proteins were found (50 and 33 in IE and OE, respectively), with 43 being absent in the shotgun approach: 22 in IE, 14 in OE, and 7 more in both membrane preparations. These results underline the complementarity of both proteomic approaches. In some cases, a peptide concentration effect in the small volume of the gel spot could facilitate detection in the 2-DE approach.

In spite of having different NCBI entries, a fair number of the identified protein species still had a certain degree of redundancy. This was mainly due to the fact that protein matches were found for similar proteins but in different plant species. Therefore, to minimize redundancy and facilitate the comparison with localizations predicted in the *Arabidopsis* At_CHLORO,[16,17] SUBA3[26] and Plant Proteome (PPDB)[27] databases, each protein species was assigned to the corresponding *Arabidopsis* ortholog (AGI nonredundant codes). Then, a nonredundant pea envelope proteome was built based on these AGI codes (Table S6 of the SI), and the corresponding Venn diagrams for the different proteomic techniques are shown in Figure 3B. A total of 409 AGIs were found using the combination of both proteomic approaches, whereas six more protein species did not have significant orthologs in *Arabidopsis*. From the total 392 AGIs identified with the shotgun approach, 229 (58%) show significant IE or OE enrichment. From these, 197 (86%) were found only with this technique, whereas 32 (14%) were also found in the 2-DE gels (15 in IE, 6 in OE and 11 in both). Regarding the 2-DE approach, 49 nonredundant proteins were found (39 and 23 in IE and OE, respectively), with 17 not present in the shotgun approach: 11 in IE, 4 in OE, and 2 in both membrane preparations (Figure 3B). In summary, the nonredundant pea envelope proteome consisted in 252 protein species, including 246 different AGIs (Figure 3B) and six more proteins without significant orthologs in *Arabidopsis* (Table S6 of the SI).

The 246 AGIs showing significant differential distribution in the two chloroplast envelope membrane preparations were first compared with the localization proposed in the At_CHLORO database (Figure 4; Table S6 of the SI). Among them, 72 were

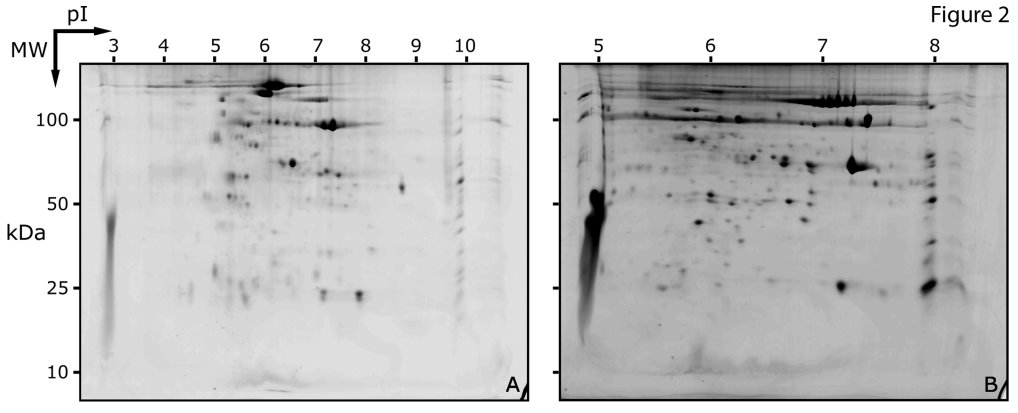

Figure 2. 2-DE IEF-SDS PAGE protein profile maps of inner (IE) and outer (OE) chloroplast preparations from pea. Proteins were separated in the first dimension in gel strips (7 cm) with a linear pH gradient (3−10 for IE and 5−8 for OE) and in the second dimension in 12% acrylamide vertical gels (8 × 10 cm) and stained with Coomassie-blue G-250. Scans of typical gels are shown for IE (A) and OE (B).

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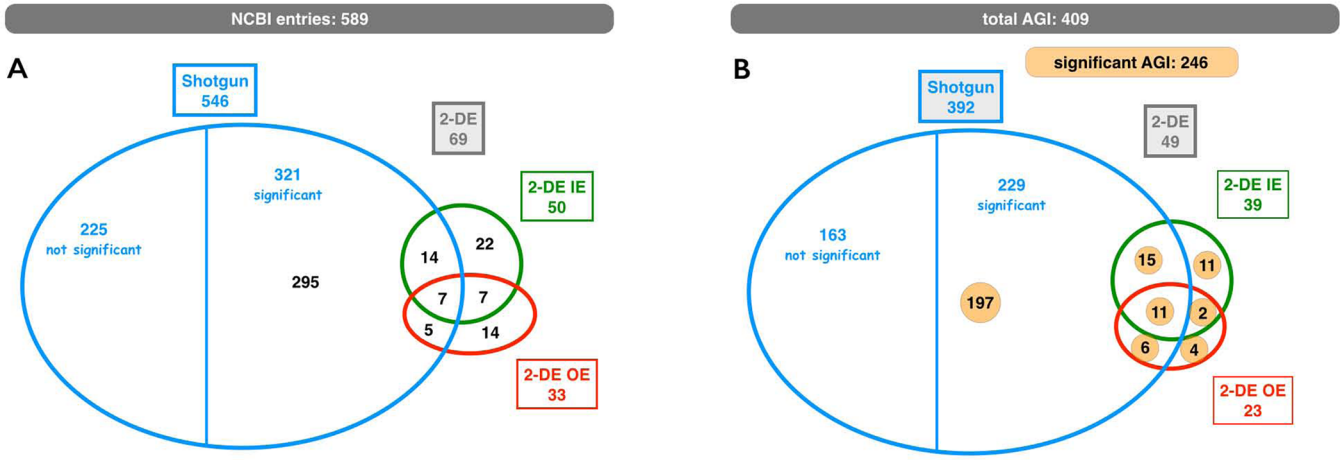

Figure 3. Venn diagrams showing partial overlaps of identified protein species between the shotgun and 2-DE techniques, using (A) the NCBI identifiers and (B) the corresponding *Arabidopsis thaliana* AGI BLAST results.

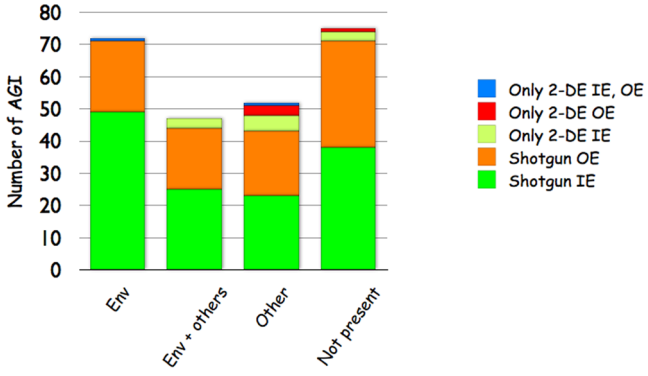

Figure 4. Localization of identified envelope proteins (using their corresponding AGI) compared to the AT_CHLORO database (16, 17) from *Arabidopsis thaliana* chloroplast proteins. A reference nonredundant orthologous *A. thaliana* proteome was built by carrying out BLAST searches of the identified (NCBI) envelope proteins of pea in the *A. thaliana* genome database (TAIR). Duplicate identifiers were deleted, and only E-values lower that e-30 were considered. Categories according to AT_CHLORO were defined as follows: Env - envelope localization; Env+Others - proteins associated with envelopes and either thylakoid or stroma; Other - proteins in nonenvelope plastid subcompartments; Not present - not found in chloroplasts.

described in the AT_CHLORO database as envelope-enriched proteins, whereas 47 were assigned to the envelope and others (thylakoid or stroma), 52 were ascribed to other subplastid compartments, and 75 more were not previously accounted for as chloroplast proteins (Figure 4 and red background entries in Table S6 of the SI). Localization was also assessed in two additional databases, PPDB and SUBA3 (Table S6 of the SI) and in general a similar plastidial localization was observed. Among the 75 proteins not present in the AT_CHLORO database, 49 were accounted for as plastidial proteins in at least one of two other databases (PPDB or SUBA3), whereas the remaining 26 were ascribed to other cell compartments or not present (Table S6 of the SI).

Delving into the IE Functions Using Shotgun Proteomics and 2-DE

Detailed functional annotation was acquired for the nonredundant pea proteome (252 protein species: 246 AGIs plus six proteins without *Arabidopsis* orthologs; Table S6 of the SI) and proteins were then classified according to their functional category (Figure 5). Transport (31 nonredundant proteins; detected only by shotgun), and proteolysis (19 nonredundant

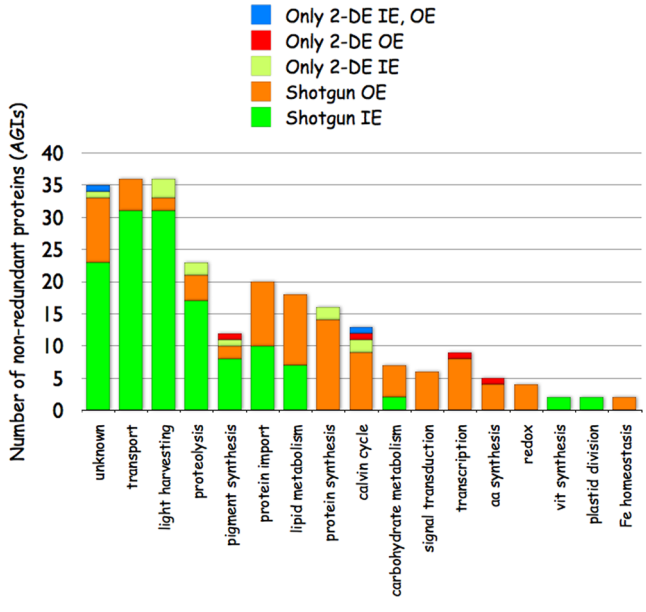

Figure 5. Distribution of nonredundant pea protein species (252 protein species: 246 AGI plus six proteins without *A. thaliana* orthologs; Table 6 of the SI) by shotgun proteomics or only by 2-DE according to their GO:P (Gene Ontology: Biological Process) annotation.

proteins; two of them only detected by 2-DE in IE gels and one detected in shotgun and by 2-DE in IE and OE) were the largest functional categories of proteins associated preferentially to the IE (Figure 5; Table S6 of the SI). Also, 27 nonredundant proteins (23 in shotgun; four detected only in 2-DE, three of them lacking AGI number) had unknown functions and higher abundances in the IE, and one more was detected by 2-DE in both IE and OE gels. These 28 proteins of unknown function were compared to the AT_CHLORO database, and from these three did not have *Arabidopsis* orthologs, eight were ascribed to the envelope, whereas one was ascribed to thylakoids. Regarding the remaining 16 AGIs not present in the AT_CHLORO database, in PPDB four were ascribed to the IE, one to envelope, one to stroma, one to thylakoid and seven

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to plastids, whereas the remaining two were not present in this database and ascribed to other cell compartments in SUBA3. The IE preparations also contained detectable traces of some highly abundant thylakoid proteins associated with photosynthetic processes, which should not be regarded as true envelope components.

The IE Has a Major Role in Transport. IE membrane preparations were significantly enriched in proteins related to transport, with 31 nonredundant proteins involved in transport of metabolites and ions and 10 more (one of them also detected by 2-DE in both IE and OE) related to the protein import machinery (in OE, only 5 and 10 proteins were found in these categories, respectively). This is indicative of a major role of the IE in active, carrier- and pump-mediated transport processes (Figure 5). Most of these proteins were already identified as envelope proteins in the AT_CHLORO and PPBD databases, with 25 already ascribed to the IE in the latter (Table S6 of the SI), and except for two of them, ABCB25 -At5g58270-, and SecA1 -At4g01800-, their localization in the IE has been either experimentally verified or is strongly predicted (ARAMEMNON plant membrane protein database[28]). Among those proteins identified, transporters for a wide array of substrates, including lipids, nucleotides, dicarboxylates, S-adenosyl methionine (SAM), and inorganic ions were detected in the IE preparations (for additional functional annotation and references, see ref 28).

Six nonredundant proteins were ABC transporters,[29] with three of them belonging to the subclass I (ABCI10 -At4g33460-, 13 -At1g65410- and 14 -At1g19800-). ABCI13 (TGD3) and ABCI14 (TGD1) participate in transfer of ER-derived lipids to the plastid galactoglycerolipid biosynthetic machinery. The function of ABCI10 is still unclear[4,29] and our results allow assigning it preferentially to the IE. The remaining three ABC transporters belong to the B subclass (ABCB 25 -At5g58270-, 26 -At1g70610- and 28 -At4g25450-). ABCB25 (ATM3/STA1) is involved in metal homeostasis (Fe−S cluster assembly and Mo-cofactor synthesis, see[28]), and it is localized in mitochondria according to the PPDB and SUBA3 databases. Since ABCB25 is annotated in envelopes by AT_CHLORO and is found in our IE preparations, the IE might be an additional subcellular localization for this transporter. The functions of ABCB26 and 28 are still unknown, but their localization to the IE is predicted in the PPDB database.[28] Three nonredundant proteins were phosphate transporters (PHT4;1 -At2g29650-, PHT2;1 -At3g26570- and PHT4;4 -At4g00370-[28]), whereas two more (PPT1 -At5g33320- and TPT -At5g46110-) were phosphoenolpyruvate (PEP)/phosphate and triose-phosphate/phosphate translocators, respectively (Table S6 of the SI). PPT1 participates in PEP supply to plastids during female and male gametophyte development,[30] and TPT is involved in the export of triose-phosphates generated in the Calvin cycle to the cytosol for sucrose synthesis.[31] Both proteins are well characterized in the IE membrane.[7,8,17] Two dicarboxylate transporters (DIT1 -At5g12860- and DIT2-1 -At5g64290-) and one protein from the major facilitator superfamily (At5g59250) involved in carbohydrate transport were also enriched in IE and allocated to the IE in PPDB.[28] Also, the recently described plastid glycerate/glycolate transporter PLGG1 -At1g32080-[32] with a function in photorespiration, not present in the AT_CHLORO database but allocated to the IE in PPDB, was identified as an IE protein as well. In addition, we found two transporters for SAM (SAMT1 -At4g39460- and SAMTL -At2g35800-), responsible for accumulation of prenyl lipids and chlorophyll biosynthesis (SAMT1).[33] While SAMT1 is already ascribed to the IE according to PPDB, our results allow localizing SAMTL preferentially to the IE. The IE preparations further contained plastid proteins of the mitochondrial carrier family such as three nucleotide transporters (AATP1/NTT1 -At1g80300-, an ATP/ADP antiporter; BT1 -At4g32400-, a putative nucleotide exporter to the cytosol, and At5g64970, with similarity to BT1[28]), as well as the protein PAPST2 -At3g51870-, a putative phosphoadenosine phosphosulfate (PAPS) transporter. The closest relative of PAPST2, TAAC/PAPST1, has recently been described to export PAPS across the IE.[34] Whereas AATP1 and ATBT1 were already ascribed to IE in PPDB, our results indicate that At5g64970 and PAPST2 are also preferentially localized to IE. RUS2 -At2g31190-, required for polar auxin transport, and PRAT2.2 -At5g24650-, belonging to the preprotein and amino acid transporter family, were also significantly enriched in IE (Table S6 of the SI). PRAT2.2 has been previously associated with a potential transport function in IE.[35]

Furthermore, the IE preparations were enriched in five cation transporters, including transporters for K (KEA2 -At4g00630-), Mg (MRS2-11/MGT10 -At5g22830-), Cu (HMA1 -At4g37270- and HMA6 -At4g33520-) and Fe (TIC21/PIC1 -At2g15290-) (Table S6 of the SI). KEA has been involved in pH and H^+/K^+ antiport,[26] PIC1 mediates Fe transport to the chloroplast[36] and the HMAs have a role in Zn detoxification in addition to Cu transport.[37] Two more nonredundant ion transporter proteins were identified: -At3g13070- similar to CorC, an Mg and Co efflux protein of *E. coli*, and MSL2 -At5g10490-, a putative mechano-sensitive ion channel involved in plastid division[28] (Table S6 of the SI). Finally, one more protein possibly involved in transport but with unknown function: -At1g31410- (not present in the AT_CHLORO database but plastid located according to the other two databases) was also more abundant in IE. All these eight proteins are plastid located in the databases used, three of them (MRS2, HMA6, and PIC1) specifically located in IE and four just described as envelope proteins; results shown here indicate that within the envelope they are preferentially allocated to the IE.

With regard to the protein import machinery, as expected, IE preparations were enriched (with relative abundances of 60−85%) in translocons, TIC22 -At4g33350-, TIC40 -At5g16620-, TIC55-II -At2g24820-, TIC55-IV -At4g25650-, TIC62 -At3g18890- and TIC110 -At1g06950-, all previously described as IE proteins[1−3] (Table S6 of the SI). Interestingly, orthologs of two components of a recently described new TIC translocon,[38] Tic214 -AtCg01130- and Tic56 -At5g01590- were also identified in IE preparations. Although these two proteins are not annotated in the AT_CHLORO dabase, TIC214 is annotated as IE and Tic56 has a plastid localization in PPDB (Table S6 of the SI). Two orthologs of the SecA pathway, SecA1 -At4g01800- and SecA2 -At1g21650-, were also enriched in IE preparations (~85%) suggesting a role of this thylakoid pathway in protein import in the IE as well.[39] Although three of the most abundant protein translocons (TIC110, TOC34, and TOC75-III) were detected by 2-DE in both IE and OE, their relative enrichments obtained by shotgun allowed for their allocation to IE (TIC110) or OE (TOC34 and TOC75-III).

Our results show a higher prevalence of transporters in IE over OE, indicating a major role of the IE in transport processes

within the envelope, but also allow for a specific IE allocation of some of them that were assigned previously only to the bulk envelope (i.e., ABCI10, SAMTL, KEA2, HMA1, PAPST2, MLS2, and PRAT2.2).

The IE Has a Potential Role in Membrane Protein Homeostasis. A total of 19 nonredundant proteins (two of them only present in 2-DE gels of IE and one protein enriched in IE in shotgun and present in 2-DE gels of both IE and OE) were plastid proteases, including members of the well-characterized families FtsH (filamentation temperature sensitive) and Clp (caseinolytic protease) (Figure 5, Table S6 of the SI). The FtsH family of membrane bound proteases was enriched in IE (12 proteins) as revealed by shotgun analysis, including: FtsH 2 -At2g30950-, 5 -At5g42270-, 7 -At3g47060-, 9 -At5g58870-, 11 -At5g53170-, 12 -At1g79560-, FtsHi1 -At4g23940-, FtsZ2-1 -At2g36250- and four putative FtsHs (-At3g02450-, -At3g04340, -At3g16290- and -At5g64580-) (Table S6 of the SI). Members of this family with one membrane-spanning region contain ATPase-AAA$^+$ type and Zn(II)-metalloprotease domains, with the exception of the chloroplast-specific FtsHi's, which lacks the Zn motif.[40] Our results support that FtsH proteins, in spite of having a major role in thylakoids, are also localized in the IE. Indeed, seven of them were annotated as envelope proteins in AT_CHLORO, whereas three more were not present in that database, one of them (FtsHi1) present in PPDB as IE[16,17] (Table S6 of the SI). FtsH5 is integrated in thylakoids via the Sec pathway, and since orthologs of SecA1 and SecA2 were also enriched in IE (~85%, see above), a similar mechanism could occur in envelopes. These proteases play a role in membrane protein maintenance, being responsible for removal of misfolded or damaged proteins,[40,41] therefore suggesting that the IE might play a role in membrane protein homeostasis.

Four nonredundant proteins of the Clp/Hsp100 family were enriched in IE as well (Table S6 of the SI). Among them, we found two of the five catalytic ClpP units of the ClpPR protease complex (1 -AtCg00670- and 4 -At5g45390-) and two chloroplast chaperones, HSP93-V/ClpC1 -At5g50920- and HASP100/ClpD -At5g51070-. Clp/Hsp100 chaperones act as independent units or with the proteolytic core to degrade irreversibly damaged proteins,[42] and are also implicated in protein import along with Hsp70 chaperones, by pulling precursors in as the transit peptide enters the organelle.[43] According to the AT_CHLORO database, these four IE Clp members are localized in the stroma and envelope[17] and in PPDB ClpC1 is located in IE, whereas the remaining three are described in the stroma (Table S6 of the SI). Clps are soluble proteins, and they have been shown to be attached to IE membranes, most likely by binding to membrane-intrinsic components of the TIC translocon complex.[43]

Two more identified proteins, the stromal processing peptidase (SPP -At5g42390-) responsible for cleavage of transit peptides[44] and a chloroplast-processing enzyme (SPPA1 -At1g73990-) were also found in our IE preparations (Table S6 of the SI). None of them has been previously reported in envelopes. SPP has been allocated in the stroma,[44] whereas SPPA1, with a yet unknown function, has been described in thylakoids[45] (Table S6 of the SI). Finally, a 17.6 kDa heat shock protein was identified only in 2-DE gels of IE membranes (Table S6 of the SI). This protein is not present in AT_CHLORO or PPDB databases and is ascribed to the cytosol in the SUBA3 database.

The IE Might Have a Role in Chlorophyll and Tocopherol Biosynthesis. While it has been previously described that the chloroplast envelope contains enzymes involved in the formation of chlorophyll (Chl) and tocopherol,[11] our results support that these processes may occur preferentially at the IE (Figure 5, Table S6 of the SI). Seven proteins of tetrapyrrole metabolism, and two of tocopherol biosynthesis were enriched in IE preparations, with abundances in the range of 70−90%. Tetrapyrrole metabolism-related proteins included two protoporphyrinogen oxidases (PPOX -At4g01690- and PPO2 -At5g14220-) from the common tetrapyrrole pathway, pheophorbide A oxygenase (PAO -At3g44880-) of the Chl breakdown pathway, and three proteins of the Chl synthesis pathway: Mg-protoporphyrin IX monomethylester cyclase (CHL27 -At3g56940-), divinyl protochlorophyllide reductase (PCB2 -At5g18660-), and Mg-protoporphyrin *O*-methyltransferase (CHLM -At4g25080-). One more, an uroporphyrinogen decarboxylase (UPD2 -At2g40490-) involved in Chl synthesis, was only detected in IE 2-DE gels (Table S6 of the SI). Four of the proteins described above had been previously ascribed to envelopes in the AT_CHLORO database,[17] with one of them (CHL27) being assigned specifically to IE in PPDB, whereas CHLM and PCB2 were not in AT_CHLORO and were broadly assigned to plastid in PPDB and SUBA3 databases, and UPD2 was assigned to the stroma in both AT_CHLORO and PPDB (Table S6 of the SI). The two proteins involved in tocopherol biosynthesis were tocopherol cyclase (VTE1 -At4g32770-) and methyltransferase (VTE4 -At1g64970-). The presence of these two enzymes in IE could be associated with plastoglobuli attached to the IE membrane, and indeed, VTE1 is assigned to plastoglobuli in PPDB. In OE, only one nonredundant protein was related to the tetrapyrrole metabolism, a glutamate 1-semialdehyde aminotransferase -At3g48730-, which was only detected in OE preparations by 2-DE and assigned to the stroma in AT_CHLORO and PPDB (Table S6 of the SI).

Envelopes are also predicted to be involved in carotenoid biosynthesis,[11,17] and three enzymes of this pathway were found in this study, a phytoene desaturase (PDS3 -At4g14210-) and a carotene monooxygenase (CYP97C1 -At3g53130-), which were more abundant in IE, and a carotenoid dioxygenase (CCD1 -At3g63520-), which was more abundant in OE (Table S6 of the SI). Although found in this study and annotated as envelope-localized in the AT_CHLORO database, these proteins might as well be ascribed to plastoglobuli attached to the envelope membranes. Indeed CCD1 was ascribed to cytosol in PPDB and vacuole in SUBA3, while PDS3 and CYP97C1 were broadly ascribed to plastids in both databases.

The IE Has a Potential Role in Lipid Metabolism. Seven nonredundant proteins ascribed to lipid metabolism had abundances in IE ranging from 62 to 85% (Figure 5, Table S6 of the SI). Identified proteins are involved in biosynthesis of fatty acids (the ACCase α-subunit CAC3 -At2g38040-, and AAE16 -At3g23790-, a putative acyl-ACP ligase), prokaryotic plastid-localized phosphatidyl glycerol (PGP1 -At2g39290-) and sulpholipids (SQD2 -At5g01220-) as well as in other lipid-modifying pathways (CYP74B2 -At4g15440-, CYP97B3 -At4g15110- and LOX6 -At1g67560-). Three of them (AAE16, CAC3, and CYP97B3) were previously described in envelopes in AT_CHLORO, with CAC3 being specifically ascribed to IE in PPDB. Among the remaining four not present in AT_CHLORO, SQD2 and CYP74B2 were ascribed to envelope and OE, respectively, in PPDB, whereas PGP1 and

LOX6 were located to plastids in either PPDB or SUBA3 databases. Interestingly, except for CYP97B3 and LOX6, at least one membrane-spanning domain is predicted for all of these proteins,[28] which points to a possible attachment of these enzymes to the IE.

The IE Preparations Contain Thylakoid Proteins. Proteins participating in the light-harvesting processes of photosynthesis were enriched (69−91%) in IE preparations (34 nonredundant proteins; three of them only detected in 2-DE gels of IE; Table S5 of the SI). When compared to the AT_CHLORO database, 21 of them were ascribed to thylakoids, nine to "thylakoids and others" including envelope, one was localized in the stroma, and four were absent in this database (Table S6 of the SI). In PPDB, all but one (ascribed to mitochondria) were localized in thylakoids (Table S6 of the SI). These results clearly indicate the occurrence of thylakoid proteins in IE preparations. All identified proteins belong to very abundant components such as the reaction centers PSI and PSII, the oxygen-evolving complex, the light-harvesting complexes LHCI and LHCII, and the chloroplast ATP synthase, and can be considered as not pertaining to the envelope (Table S6 of the SI). Among all the proteins in this category, there is experimental evidence of IE localization for only one protein, the ferredoxin-NADP reductase (FNR, -At5g66190-, detected only by 2-DE in IE preparations). This protein, besides binding to thylakoids, interacts with the IE via TIC62.[46]

In contrast, the presence of thylakoid proteins in OE was lower than in IE preparations. Only two proteins, the subunit D1 of PSI RC (PSAD1) and the subunit R of the PSII RC (PSBR), were enriched in OE preparations as revealed by shotgun, and none was detected only in 2-DE (Table S6 of the SI). The presence of thylakoid proteins in the IE preparations is inherent to the isolation method used to obtain envelopes. The osmotic shock occurring during chloroplast isolation can likely cause an attachment of traces of thylakoids to IE membranes and therefore explain the presence of thylakoid proteins, especially in the IE preparations.[12]

Delving into the OE Composition by Shotgun Proteomics and 2-DE

While IE membrane preparations were clearly enriched in proteins functioning in transport and protein homeostasis, proteins preferentially associated with OE participate in a wider range of processes (Figure 5). The largest functional categories within the nonredundant proteins preferentially associated with OE were those related to transport, including protein import (10 proteins) and lipid metabolism (11 proteins; one only detected by 2-DE in OE). Other categories were carbohydrate metabolism (four proteins), redox homeostasis and proteolysis (with four proteins, each) and signal transduction (six proteins). This diverse spectrum of pathways can be explained because processes occurring at the OE involve association or membrane-anchoring of proteins to the cytosolic face of the envelope. Twelve nonredundant proteins (10 revealed by shotgun and two found only in 2-DE) had unknown functions, and from these, two did not have *Arabidopsis* orthologs, three were ascribed to the envelope in AT_CHLORO, one was ascribed to IE in PPDB, two more were ascribed to plastid in PPDB or SUBA3, whereas the remaining four were not present in AT_CHLORO or PPDB or ascribed to other compartments in SUBA3. However, more than 50% of the proteins absent in the AT_CHLORO database were predicted to contain a membrane-anchor domain.

In general, most of the proteins targeted to the OE do not contain a classical, N-terminal plastid signal sequence and membrane-spanning domains of channel pores are often built by β-sheets, which arrange to a β-barrel structure.[2,6] Therefore, *in silico* prediction of OE proteins is difficult, in contrast to the IE, where most of the precursor proteins are targeted via N-terminal signal peptides and membrane-intrinsic proteins preferentially consist of hydrophobic, α-helical domains. Potential OE-membrane-anchoring domains of proteins are discussed thereafter as listed in ARAMEMNON.[28]

The OE Roles in Transport. In contrast with the abundance of this protein class in IE, only five nonredundant proteins related to transport were enriched in OE (Figure 5, Table S6 of the SI). These included an ABC transporter of subfamily G (ABCG7 -At2g01320-), and the already well characterized "outer envelope proteins" (OEPs), which form channel pores for metabolites: OEP16.1 (-At2g28900-, specific for amino acids), OEP21.1 (-At1g76405-, transporting phosphorylated carbohydrates and phosphate), and OEP24.1 (-At1g45170-) and OEP37 (-At2g43950-), both most likely being channels for small solutes.[6,35,47−50] Even though OEP24.1 is not present in AT_CHLORO, in PPDB it is classified as an OE protein (Table S6 of the SI).

OE preparations were enriched (with relative abundances of 80−99%) in eight nonredundant proteins belonging to the OE translocon for protein import (Figure 5, Table S6 of the SI). These included the well-known translocon proteins TOC34 -At5g05000-; TOC64-III -At3g17970-; TOC75-III -At3g46740-; TOC75-V -At5g19620-; TOC90 -At5g20300-; TOC120 -At3g16620-; TOC132 -At2g16640-; and TOC159 -At4g02510-.[1−3] Among these, TOC34 and TOC75-III were detected by 2-DE in both IE and OE preparations. Two members of the HSP70 chaperon family (HSP-70 -At3g12580- and HSP70-1 -At5g02500-), with potential functions in protein translocation[43] were found preferentially attached to OE membranes as well, although HSP-70 was also detected by 2-DE in IE preparations. Whereas the enrichment of OE and IE translocon compounds is clearly shown by the shotgun proteomic approach, residual OE/IE cross-contamination with some very abundant proteins (i.e., TIC110 in OE, TOC75 in IE) is still visible in the 2-DE analysis (Table S6 of the SI).

The OE Roles in Lipid Metabolism. Twelve nonredundant proteins were related to fatty acid/lipid metabolism, including 10 proteins with high abundances in OE (67−97%), one only detected by 2-DE in OE and one with higher abundance in OE, but also detected by 2-DE in IE (Figure 5, Table S6 of the SI). Some of the identified proteins are involved in fatty acid synthesis, including the three components of the pyruvate dehydrogenase complex (pyruvate dehydrogenase β-subunit E1β -At2g34590-, two dihydrolipoamide acetyl transferases E2 -At1g34430- and -At3g25860-, lipoamide dehydrogenase E3 -At3g16950-), an ACCase subunit of biotin carboxylase (CAC2/BC -At5g35360-), and a long-chain fatty acid synthase (LACS9 -At1g77590-). Also, a protein similar to dihydrolipoamide acetyl transferase without an *Arabidopsis* ortholog was only found by 2-DE in OE preparations (Table S6 of the SI). Among them, LACS9 is ascribed to envelopes in the AT_CHLORO databases, and accordingly, it has been described to be most likely attached to the OE,[51] whereas CAC2 was ascribed to stroma (in AT_CHLORO) or IE (in

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PPDB), and the subunits of the pyruvate dehydrogenase were either ascribed to "envelope and others" or absent in AT_CHLORO or broadly ascribed to plastids in the other two databases (Table S6 of the SI). Other proteins involved in lipid metabolism enriched in our OE preparations were PECT1 (-At2g38670- involved in eukaryotic phospholipid synthesis), the β-glycosidase SFR2 [-At3g06510- a galactolipid remodeling enzyme of the OE membrane, involved in freezing tolerance,[52] PAP (-At3g26070-, a lipid associated protein), a potential dephospho CoA kinase (ATCOE -At2g27490-), and a cytochrome *b*5 reductase (CBR1 -At5g17770-) that provides electrons for fatty acid desaturases.[53] In the AT_CHLORO and PPDB databases PAP is ascribed to thylakoids and SFR2 to OE, whereas ATCOE is localized in the envelope in AT_CHLORO and in the peroxisome in the other two databases. The remaining, PECT1 and CBR1, are not present in AT_CHLORO and are assigned to compartments other than plastids in the other two databases (Table S6 of the SI). It should be noted that PECT1, CBR1, and ATCOE have a predicted α-helical membrane-domain,[28] that could anchor these enzymes to the cytosolic face of the OE. Together with the fatty acid/lipid-associated proteins found at the IE (see Figure 5), these results indicate that both membranes have an active role in lipid metabolism.

A Potential Role of the OE in Carbohydrate Metabolism. Five nonredundant proteins involved in carbohydrate metabolism were also more abundant (61−97%) in OE (one of them being detected by 2-DE in both IE and OE, Figure 5, Table S6 of the SI). These included two hexokinases (HXK1 -At4g29130- and HXK2 -At2g19860-), a transaldolase of the pentose phosphate shunt -At1g12230-, an aldehyde dehydrogenase -At1g44170-, and a carbonic anhydrase (BCA1 -At3g01500-). Among them, HXK1, the transaldolase and BCA1 are ascribed to the envelope in AT_CHLORO, whereas HXK2 and ALDH4 are not present and ascribed to mitochondria or cytosol in the other two databases (Table S6 of the SI). Interestingly, spinach HXK1 can insert into the OE via an N-terminal membrane anchor,[54] most likely energizing glucose export from plastids. With regard to the three proteins absent in AT_CHLORO, only ALDH4 contains a predicted membrane domain[28] that could attach this protein to the OE. In contrast to OE, only two proteins related to carbohydrate metabolism were more abundant in IE: a plastid-NAD-dependent malate dehydrogenase (-At3g47520-) and a Golgi-localized xyloglucan galactosyltransferase (KAM1 -At2g20370-).

The OE Contains Proteins Related to Proteolysis and Redox Defense. In contrast to the abundance of proteins related to proteolysis in the IE, only four nonredundant proteins of this class were found enriched in OE preparations (Figure 5, Table S6 of the SI). These included ClpP5 -At1g02560-, two metallo-endopeptidases (-At5g56730- and -At1g67690-) and polyubiquitin (UBQ11 -At4g05050-). In AT_CHLORO, Clp5 and -At5g56730- are ascribed to the envelope, whereas -At1g67690- and UBQ11 are not present and ascribed to nucleus, mitochondria, or plasma membrane in the other two databases. However, the metalloprotease At1g67690 contains a membrane-anchor domain[28] that supports its presence also in OE.

Nonredundant proteins involved in redox homeostasis were only found by shotgun in OE, with abundances ranging from 77 to 95% (Table S6 of the SI). These included two peroxiredoxins (2CPB -At5g06290- and PEX22 -At3g21865-), ascorbate peroxidase (APX3 -At4g35000-) and monodehydroascorbate reductase (MDAR4 -At3g27820-). APX3 is localized in the envelope in the AT_CHLORO database, whereas 2CPB is assigned to the stroma in both AT_CHLORO and PPDB, PEX22 is assigned to the nucleus in SUBA3, and MDAR is assigned to the peroxisome and plastid in PPDB and SUBA3, respectively. However, PEX22 contains an anchor domain[28] that may support its presence in OE.

An OE Role in Signal Transduction. Six nonredundant proteins, with abundances between 80 and 93% in OE, are related to signal transduction (Table S6 of the SI). Among them, phototropin 2 (PHOT2 -At5g58140-) is a blue light photoreceptor involved in chloroplast movement that has been recently localized in OE,[55] and WAV2 (-At5g20520-), is a membrane-bound protein involved in modulation of root bending,[56] not present in AT_CHLORO or PPDB and assigned to ER in SUBA3. The remaining four proteins belong to the rho (MIRO1 -At5g27540- and MIRO2 -At3g63150-) and rab (RAB8A-like -At5g59840-, RAB1B -At4g17170-) subfamilies of the small GTPase superfamily. In the AT_CHLORO database only RAB1B is ascribed to the envelope, whereas the other three are absent and ascribed to other cell compartments in the PPDB and SUBA3 (Table S6 of the SI).

Stroma-Localized Proteins Detected in OE Preparations. Several functional categories (Calvin cycle, ribosomal proteins, amino acid synthesis, transcription, and Fe homeostasis) of soluble proteins typically found in the stroma were enriched in our OE preparations. As commented above for the presence of thylakoid proteins in IE preparations, the presence of stroma-localized proteins in OE preparations is most likely facilitated by the osmotic shock during chloroplast isolation that could trap stromal proteins inside OE vesicles.[12] In addition, charges at the membrane and protein surface levels might favor soluble proteins preferentially attaching to OE.

Ten nonredundant proteins (five of them also detected by 2-DE, three in both IE and OE, and two in IE) and one more protein, detected only by 2-DE in both IE and OE, were related to the Calvin cycle (Figure 5, Table S6 of the SI). In contrast, shotgun results did not reveal any protein in this category enriched in IE and only two nonredundant proteins were found by 2-DE. When compared to the AT_CHLORO database, eight of them were ascribed to the stroma, whereas five were localized in both envelope and stroma (Table S6 of the SI).

Amino acid metabolism (five nonredundant proteins with one detected only by 2-DE), Fe homeostasis (two nonredundant proteins), and transcription (nine proteins with one detected only by 2-DE) were also among functional categories containing proteins with high abundance in OE. When compared to the databases, identified proteins involved in amino acid and Fe metabolism were ascribed to the stroma or plastid (Table S6 of the SI), whereas those related to transcription were mostly assigned to the plastid nucleoid or the nucleus, with PTAC16 (-At3g46780-) being the only protein ascribed to envelopes in the AT_CHLORO database. None of these proteins contained membrane-anchoring domains, thus favoring the stromal origin of these proteins in OE preparations. Finally, 14 nonredundant proteins involved in protein synthesis were also found to be preferentially located in OE by shotgun (with two of them also detected by 2-DE in both IE and OE preparations), whereas only two nonredundant proteins were detected in this category by 2-DE in IE (Figure 5, Table S6 of the SI). This group contained, among others,

different constituents of the 30S and 50S plastid ribosomal subunits and two elongation factors (Table S6 of the SI). In the AT_CHLORO database, seven of these 14 proteins were localized to the envelope and stroma, whereas five were ascribed only to the stroma, and two more were not present, indicating again the presence of stromal proteins in the OE. In PPDB, all were assigned to the plastid ribosome except for one, the peptidyl-tRNA hydrolase PTH2 (-At5g16870-), predicted in the cytosol and with a potential α-helical membrane-anchor.[28] For the ribosome-associated endonuclease CSP41B/HIP1.3 (-At1g09340-),[57] OE attachment appears to be possible. Interestingly, for CSP41B/HIP1.3 a function as heteroglycan-interacting protein has been proposed,[58] with the protein being associated with the cytosolic surface of chloroplast OE membranes.

CONCLUSIONS AND FINAL REMARKS

In summary, our combined proteomic approach has allowed further progress toward the characterization of the protein components of the inner (IE) and outer (OE) chloroplast envelope membranes of pea. Envelope preparations not only contain membrane-intrinsic and membrane-attached proteins, but also a certain degree of contamination from neighboring compartments, mainly corresponding to very abundant thylakoid or stroma proteins in IE and OE, respectively. In this study we have used the relative enrichment of each protein in the IE/OE pair of membranes to provide an integrated picture of the actual composition and dynamics of the system. The IE is clearly enriched in proteins related to transport and protein metabolism, whereas proteins preferentially associated with OE are more heterogeneous and participate in a wider range of processes. Relative enrichments clearly indicate that in the envelope, the IE is a selective barrier for the transport of a wide range of metabolites and ions, and also plays a major role in controlling membrane protein homeostasis, given the high prevalence of metallo-proteases involved in the removal of damaged proteins. Furthermore, based on our data, metabolic processes previously described to occur in the envelope, including chlorophyll and tocopherol biosynthesis, can be ascribed preferentially to the IE, whereas others such as fatty acid and lipid synthesis occur in both the IE and OE.

Also, whereas results show a good overlap with their previous annotation in the AT_CHLORO and other plant protein databases, they add a new layer of information by allowing further assignation of envelope proteins specifically to the IE and/or the OE. Furthermore, this combined approach has revealed 26 nonredundant proteins (Table S6 of the SI) in envelope preparations that had not been previously found in the chloroplast according to the databases used in the analysis. However, the final assignment of these proteins to the envelope needs further experimental evidence. Finally, it should be mentioned that even though shotgun is a technique more powerful than 2-DE for membrane protein profiling, both appear to be complementary, with many proteins being detected in only one of the two methodologies.

ASSOCIATED CONTENT

Supporting Information

Table S1: Full list of peptides identified and quantified by shotgun proteomics and Progenesis LC−MS analyses; Table S2: List of peptides found by MALDI-TOF MS, and LC−MS/MS in the NCBI protein species identified with the 2-DE approach; Table S3: List of protein species showing significant differences in abundance between IE and OE membrane preparations (ANOVA, $p < 0.05$) using shotgun proteomics; Table S4: List of protein species showing no significant differences in relative abundance between IE and OE membrane preparations (ANOVA, $p < 0.05$) using shotgun proteomics; Table S5: List of protein species identified in the different spots of 2-DE IEF-SDS PAGE gels; Table S6: Nonredundant proteome of the pea chloroplast envelope. Figure S1: Representative one-dimensional SDS-PAGE gel of the IE and OE preparations used for proteomic analyses; Figure S2: Immunoblot analysis of seven representative proteins identified and quantified in the shotgun proteomic approach. Figure S3: Virtual composite images containing all spots present in the real 2-DE IEF-SDS PAGE gels are shown in (A) for IE and (B) for OE. This material is available free of charge via the Internet at http://pubs.acs.org..

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Notes

The authors declare no competing financial interest.

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