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Tocopherol Cyclase (VTE1) Localization and Vitamin E Accumulation in Chloroplast Plastoglobule Lipoprotein Particles*

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Chloroplasts contain lipoprotein particles termed plastoglobules. Plastoglobules are generally believed to have little function beyond lipid storage. Here we report on the identification of plastoglobule proteins using mass spectrometry methods in Arabidopsis thaliana. We demonstrate specific plastoglobule association of members of the plastid lipid-associated proteins/fibrillin family as well as known metabolic enzymes, including the tocopherol cyclase (VTE1), a key enzyme of tocopherol (vitamin E) synthesis. Moreover, comparative analysis of chloroplast membrane fractions shows that plastoglobules are a site of vitamin E accumulation in chloroplasts. Thus, in addition to their lipid storage function, we propose that plastoglobules are metabolically active, taking part in tocopherol synthesis and likely other pathways.

Chloroplasts are highly compartmentalized organelles. In addition to membrane-bound compartments, chloroplasts contain lipoprotein particles called plastoglobules. Although a role of plastoglobules in chloroplast differentiation has been defined (1, 2), the functions of plastoglobules in chloroplasts are largely unknown. Plastoglobules are associated with thylakoid membranes (3), suggesting that they play a role in thylakoid membrane function. Indeed, plastoglobules enlarge during thylakoid disassembly in senescing chloroplasts and during chloroplast disassembly (4–10) and increasingly accumulate triacylglycerols and esterified isoprenoids derived from the disintegrating thylakoids (6, 11). Plastoglobules have been reported to contain prenylquinones tocopherol and plastoquinone (8, 11–15), but the relative abundance of these compounds with regard to other chloroplast compartments is unknown. Although tocopherols are thought to function as antioxidants mostly at the thylakoid membrane, most of the prenylquinone biosynthetic activities have been localized to the inner chloroplast envelope membrane (16–18).

Pea plastoglobules contain around a dozen different proteins (3) but so far only members of the plastid lipid-associated protein (PAP)/fibrillin family have been identified (1, 3, 19–28). At least two PAP/fibrillins were localized at the periphery of plastoglobules (3, 26), and purified fibrillin was able to promote carotenoid fibril assembly in vitro (1), suggesting a structural function (29). Upregulation of several PAP/fibrillins has been correlated with various treatments generating reactive oxygen species (20, 24, 30–32), and enlarged plastoglobules have been described in chloroplasts under abiotic stress such as drought (33), nitrogen starvation (34), or hyperosmality (35). Taken together, these observations implicate plastoglobules in stress tolerance.

In addition to a role in plastid lipid storage and a possible involvement in stress tolerance, plastoglobules may have other functions in chloroplasts. The presence of yet-unidentified proteins of plastoglobules (3) supports this idea.

To identify candidate plastoglobule proteins we used mass spectrometry methods. We found structural proteins of the PAP/fibrillin family as well as metabolic enzymes. In particular, we show that VTE1 (tocopherol cyclase) is localized at plastoglobules and demonstrate that plastoglobules are a site of vitamin E accumulation in chloroplasts. The results imply that plastoglobules do not only store vitamin E but actively participate in its synthesis.

EXPERIMENTAL PROCEDURES

Isolation and Fractionation of Intact Chloroplasts—Intact chloroplasts were isolated from 6–8-week-old Arabidopsis thaliana (Columbia 2) plants grown on soil under short days (8 h light) and standard conditions (130 μmol of photons m⁻² s⁻¹, 20–22 °C, 80% relative air humidity) according to the protocol described in Hiltbrunner et al. (36). Chloroplasts corresponding to 40 or 60 mg of chlorophyll were hyperonically lysed in 0.6 M sucrose. To prevent proteolytic degradation, 0.5% (v/v) protease inhibitor mixture for plant cell extracts (Sigma) was added to the lysate. The stroma was separated from total membranes by centrifugation at 100,000 × g. The membrane pellet was resuspended in 10 ml of 45% sucrose in TED buffer (50 mM Tricine, pH 7.5, 2 mM EDTA, 2 mM dithiothreitol) using a Potter homogenizer. Total membrane fraction (corresponding to 20–30 mg of chlorophyll) was then overlaid with a discontinuous sucrose gradient consisting of 6 ml of 38% sucrose, 6 ml of 20% sucrose, 4 ml of 15% sucrose, and 8 ml of 5% sucrose.
in TED buffer and centrifuged for 17 h at 100,000 × g and 4 °C (SW28Ti rotor, Beckman Instruments). 1-ml fractions were collected starting from the top of the gradient.

**Protein Identification by Mass Spectrometry**—Plastoglobule-containing fraction 1 was subjected to SDS-PAGE, and proteins were stained with silver nitrate (modified from Blum et al. (37)). Visible protein bands were excised and subjected to in-gel tryptic digest, essentially as described (38). Before identification by mass spectrometry, tryptic peptides were separated by reversed phase liquid chromatography on a C18 resin. Peptides were separated on laboratory-made silica capillary columns with an inner diameter of 75 μm (length 8 cm). Five μl of peptides resolved in buffer A (5% acetonitrile, 0.5% formic acid, or 0.5% acetic acid in Millipore water) were loaded onto the column and eluted with an increasing concentration of acetonitrile (buffer B: 0.5% formic acid or 0.5% acetic acid in acetonitrile) at a flow rate of ~300 nl/min. Complex peptide mixtures were separated by increasing the acetonitrile concentration from 5 to 65% over 2 h followed by an increase of up to 85% for an additional 15 min. For less complex mixtures a shorter gradient of 40 plus 10 min was developed. Reversed phase liquid chromatography was coupled online to an LCQDecaXP ion trap mass spectrometer (Thermo Finnigan, San Jose, CA) equipped with a nanospray source. Analysis was performed in the positive ion mode, and peptides were ionized with a spray voltage of 2.1–2.8 kV and analyzed by one MS full scan and four data dependent MS/MS scans of the four most intense parent ions. The dynamic exclusion function was enabled to allow two measurements of the same parent ion for 1 min following by exclusion duration of 1 min (39).

MS/MS data were interpreted by the SEQUEST software (Thermo Finnigan) searching the data against either the *A. thaliana* protein data base or the NCBI non-redundant protein data base (www.ncbi.nlm.nih.gov). Cysteines were allowed to be either unmodified or carboxy-amidomethylated. Protein identification was accepted if two different peptides exceeded an Xcorr value of 2.5 and a ΔCn (change in correlation normalized) value of 0.1.

**Transient Expression of Green Fluorescent Protein (GFP), CFP, and YFP Fusion Proteins in *A. thaliana* Protoplasts**—AtPGL35 and AtPGL34 cDNA clones (U16632 and U15686, respectively) were obtained from the Arabidopsis Biological Resource Centre (www.arabidopsis.org/abrc). All other cDNAs were isolated by reverse transcription-PCR performed on RNA extracted from leaves. Complete coding sequences excluding the stop codon were amplified by PCR using 5’ and 3’ primers including Ncol (or XbaI and BspHI) sites (AtPGL30.4, 5’-GCC ATG CCA TGG CAG CAA TGA CGA ATA CCC-3’; AtPGL34, 5’-CAT GCC ATG GCA TGG ATC ATA CAA CAT GG-3’; AtPGL35, 5’-CAT GCC ATG GCG ACG GTA CCA TTG-3’; AtPGL36, 5’-CAT GCC ATG GCA TGG TTA AAG AAG GAG CCT TC-3’; AtAOS, 5’-TCC ATG CCA TGG CCT CTT CTA CAA CCC-3’; 5’-CCA TGC CAT GGC AAA GCT AGC TTC CTA CAA C3-3’; AtPGL37, 5’-GCC ATG CCA TGG CAG CAA TGA CGA ATA CCC-3’; AtPGL38, 5’-GCC ATG CCA TGG CAG CAA TGA CGA ATA CCC-3’; AtPGL39, 5’-GCC ATG CCA TGG CAG CAA TGA CGA ATA CCC-3’). PCR products were ligated into the Ncol (or XbaI and Ncol) sites of pCL60 (40), resulting in C-terminal GFP fusions under the control of the cauliflower mosaic virus 35S promoter and the nos terminator. For co-localization in protoplasts, AtPGL35, AtVTE1, and AtFBA2 coding sequences were cloned in pCL62 or pCL61. pCL62 and pCL61 (Dr. Andreas Hilbrunner, Institute for Biology, Albert-Ludwigs-Universität Freiburg) are derivatives of pCL60 with CFP and YFP replacing GFP, respectively. *A. thaliana* Col. 2 plants used for protoplast isolation were grown for 3–4 weeks on 0.5× Murashige-Skoog medium under long day conditions (16 h light). Transient transformation of protoplasts was done using the polyethylene glycol method as described (41) but reducing cellulase and macerozyme (Serva, Heidelberg, Germany) concentrations to 1% and 0.25% (w/v), respectively.

Fluorescence in transformed protoplasts was monitored 48 to 80 h after transformation by confocal laser scanning microscopy. The fluorescein isothiocyanate (488 nm) laser line from a Leica TCS 4D microscope (Leica Microsystems, Glattbrugg, Switzerland) was used to detect GFP. For double fluorescent experiments, CFP and YFP were detected sequentially using 458- and 514-nm laser lines as well as 460–510- and 520–588-nm detection windows from a Leica SP2 AOBS microscope. Chlorophyll autofluorescence was monitored using either 594-nm or TRITC (568-nm) excitation wavelengths.

**Protein Analysis**—Total proteins were isolated from *Arabidopsis* leaves according to Rensink et al. (42). Protoplasts transiently expressing GFP, CFP, and YFP fusion proteins were centrifuged for 1 min at 100 × g and resuspended by vortexing in extraction buffer (100 mM NaCl, 50 mM Tris-HCl, pH 7.5, 0.5% (v/v) Triton X-100, 10 mM β-mercaptoethanol) supplied with protease inhibitor mixture (Sigma P9599). Proteins were concentrated by chloroform–methanol precipitation (43), separated by SDS-PAGE, and either stained by silver nitrate or blotted onto nitrocellulose membrane for immunodetection.

Blots were probed with a serum raised against GFP (kindly provided by Prof. E. Schäfer, Institute for Biology, Albert-Ludwigs-Universität Freiburg, Germany), AtAOS (kindly provided by Dr. I. Kubigstelig (44)), chlorophyll a/b-binding protein (CAB; kindly provided by Dr. K. Apel, ETH Zurich, Switzerland), Rubisco large subunit (kindly provided by Dr. S. Crafts-Brandner, United States Department of Agriculture, Phoenix, AZ), AtVTE1 (45) or AtTIC110 (46), or with affinity-purified antibodies specific for AtTOC75 (36) and AtPGL35 (this paper). Chlorophyll concentration was determined by the method of Arnon (47), and proteins were quantified following the Bradford method (48).

**Immunolocalization in Protoplasts**—Immunolocalization in isolated protoplasts was performed as described in Matsui et al. (49) with the following modifications. Protoplasts were fixed with 4% (w/v) formaldehyde in W5 buffer (154 mM NaCl, 125 mM CaCl2, 5 mM KCl, 5 mM glucose, 1.5 mM MES, pH 5.6) and permeabilized with 0.5% (v/v) Nonidet P-40 in W5. Primary antibodies were diluted 1:50 (v/v, anti-CAB serum) and to 15 ng/μl (anti-AtTOC159 and anti-AtPGL35). Slides were mounted in SlowFade solution (Molecular Probes, Basel, Switzerland), and fluorescence of the fluorescein–coupled secondary antibody (Pierce) was monitored by confocal scanning microscopy using the fluorescein isothiocyanate (488 nm) laser line from a Leica TCS 4D microscope (Leica Microsystems).

**Preparation of Anti-AtPGL35 Antibody**—The coding sequence of AtPGL35 was subcloned from pCL60-AtPGL35 (see above) in pET21d-Ha, pET21d-Hb was obtained by digesting pET21d (Novagen) with Ncol and EcoRi and inserting a hexahistidine tag in-frame with the Ncol site using the primers 5’-CAT GCC TGA CCA TGG CAG CAA TGA CGA ATA CCC-3’; 5’-TCC ATG CCA TGG CAG CAA TGA CGA ATA CCC-3’; 5’-GCT ATG CCA TGG CAG CAA TGA CGA ATA CCC-3’; 5’-CAG ATG CCA TGG CAG CAA TGA CGA ATA CCC-3’. PCR products were ligated into the Ncol (or XbaI and Ncol) sites of pCL60 (40), resulting in GFP fusions under the control of the cauliflower mosaic virus 35S promoter and the nos terminator. For co-localization in protoplasts, AtPGL35, AtVTE1, and AtFBA2 coding sequences were...
recommendations. Polyclonal antibodies were produced in rabbit (Eurogentec, Seraing, Belgium) and were affinity-purified against recombinant AtPGL35 coupled to Affi-Gel 10 (Bio-Rad).

**Electron Microscopy and Immunocytochemistry**—Cotyledons from 5-day-old *A. thaliana* (Landsberg erecta) plants grown on MS-sucrose (Murashige and Skoog medium plus sucrose) medium were excised and transferred to aluminum sample holders, cryo-protected with 150 mM sucrose, and frozen in a Baltec HPM 010 high pressure freezer (Technotrade, Manchester, NH). Samples were then freeze-substituted in 2% OsO4 in anhydrous acetone at −80 °C for 5 days followed by slow warming to room temperature over a period of 2 days, removed from the holders, and infiltrated with increasing concentrations of Epon (Ted Pella, Inc., Redding, CA). Polymerization was carried out at 60 °C for 2 days under vacuum.

For protein immunogold labeling, the high pressure frozen samples were substituted in 0.2% uranyl acetate plus 0.25% glutaraldehyde in acetone at −80 °C for 5 days and warmed to −60 °C for 18 h. After several acetone rinses, samples were removed from the holders and slowly infiltrated at −60 °C with Lowicryl HM20 (Electron Microscopy Sciences, Fort Washington, PA) for 1 day each in 10, 25, and 75 HM20 in anhydrous acetone. For 3 more days, 100% HM20 was used and was replaced with freshly made resin every 8 h. Finally, samples were polymerized at −60 °C under UV light for 72 h. Freeze substitution, embedding, and resin polymerization were carried out under controlled time and temperature conditions in a Leica AFS system (Vienna, Austria).

**Immunocytochemistry**—Samples embedded in Lowicryl HM20 were sectioned into 100-nm-thick sections and placed on Formvar-coated gold slot grids. Immunocytochemistry was performed essentially as described (50). Briefly, the sections were blocked for 20 min with a 5% (w/v) solution of nonfat milk in TBS plus 0.1% Tween 20 (TBST). Primary anti-AtPGL35 and anti-AtVTE1 antibodies were diluted 1:20 in a solution of 2.5% nonfat milk in TBST at room temperature for 1 h. The sections were rinsed in a stream of TBS plus 0.5% Tween 20 and then transferred to the secondary antibody (anti-rabbit IgG conjugated to 10-nm gold particles, 1:20 in TBST) for 1 h. Control procedures were performed by omitting the primary antibody.

**Tocopherol and Fatty Acid Analysis**—For tocopherol and fatty acid analysis, fractions of sucrose gradient were pooled as follows; fractions 1–4 (referred to as plastoglobule fraction), 5–13 (referred to as in between fraction), 14–18 (referred to as outer envelope membrane/thylakoid fraction), 19–26 (referred to as outer- inner envelope membrane/thylakoid fraction), and 27–31 (thylakoid/envelope membrane fraction). Tocopherol was extracted with diethyl ether and quantified by normal phase fluorescence HPLC using tocol as internal standard (51). Gradient fractions were acidified by adding ½ volume of 1 M KCl, 0.2 N H3PO4, and lipids were extracted 3 times with 2 volumes each of chloroform/methanol (2:1). The organic phases were combined, and the solvent was evaporated in a nitrogen gas stream. Fatty acids were quantified by gas chromatography with pentadecanoic acid (15:0) as the internal standard according to Browse et al. (52).

**RESULTS**

**Characterization of an Arabidopsis Plastoglobule Marker Protein**—We have previously identified PG1 (plastoglobulin 1) as a major protein associated with plastoglobules in pea (3). In *Arabidopsis*, 13 proteins share sequence similarity with pea PG1, constituting the family of PAP/fibrillins as described by Laizet et al. (23). Because PAP and FIB have already been defined by the International Arabidopsis community (TAIR; www.arabidopsis.org) as standing for phosphatidic acid phosphatase and fibrillarin, respectively, we propose to abbreviate Arabidopsis PAP/fibrillins as plastoglobulin (PGL). To obtain a marker for *Arabidopsis* plastoglobules, we produced affinity-purified antibodies against a recombinant *Arabidopsis* protein AtPGL35 (At4g04020), highly similar to pea PG1. In a total protein extract of *Arabidopsis* leaves, the anti-AtPGL35 antibodies recognized a single band (Fig. 1A) of the expected mass (~35 kDa).

Immunogold electron microscopy was used to localize AtPGL35 in 1-week-old *Arabidopsis* cotyledons (Fig. 1B). Labeling was restricted to chloroplasts. Gold particles were detected at the periphery of negatively stained plastoglobules but were absent from thylakoid or envelope membranes. No labeling was found in control experiments where affinity-purified anti-AtPGL35 antibody was omitted (data not shown). The data indicate that AtPGL35 is associated with plastoglobules. The anti-AtPGL35 antibody gave dot-like fluorescence in immunofluorescence localization of AtPGL35 in cotyledons. Sections of *Arabidopsis* cotyledons were incubated with anti-PGL35 antibodies followed by secondary gold-conjugated antibodies and observed using transmission electron microscopy. p, plastoglobules; g, thylakoid grana. C, fluorescent immunolocalization of AtPGL35 in protoplasts. Fixed protoplasts were probed with anti-AtPGL35, anti-AI0C159, or anti-CAB antibodies as indicated or directly incubated with the fluorescein-labeled secondary antibody (control). The detection of the chromophore fluorescence (fluorescein) by confocal microscopy, transmission microscopy images (transmission), and merged fluorescence and transmission images (merge) are shown. Bar length, 5 μm.
experiments using fixed protoplasts (Fig. 1C). The fluorescent dots are consistent with the immunofluorescent labeling of plastoglobules. Control antibodies against AtTOC159 (At3g46740) and CAB resulted in rim-like fluorescence at the envelope or fluorescence at the thylakoids, respectively.

Isolation of A. thaliana Plastoglobules—We have previously described the isolation by flotation centrifugation of plastoglobules from pea chloroplasts (3). Here, we adapted the method to isolate plastoglobules from Arabidopsis chloroplasts. Total membranes of isolated chloroplasts were separated from stromal proteins by centrifugation at 100,000 × g. To separate plastoglobules from chloroplast membranes, the membrane pellet was resuspended in a 45% sucrose solution and overlaid with a sucrose step gradient consisting of 38, 20, 15, and 5% sucrose solutions and centrifuged overnight at 100,000 × g. This resulted in an apparent separation of membrane fractions (Fig. 2A) corresponding to broad and partially overlapping peaks of marker proteins in Western blotting analysis (Fig. 2C).

Antibodies against the CAB protein gave strong signals in fractions 19–31 (Fig. 2C), indicating the presence of thylakoid membranes in the high density 45 and 38% sucrose steps. Weaker CAB signals in fractions 1–13 were most likely due to a minor presence of highly abundant thylakoid membranes. The RuBisCO large subunit was detected in the stromal fraction and more weakly in fractions 21–31, indicating the absence of stromal contamination in the lower density fractions. AtTOC75, a protein of the outer envelope membrane, was detected in fractions 11–31. Similarly, the inner envelope membrane protein AtTIC110 (At1g06950) was detected in fractions 19–29 (Fig. 2C). The lower density fractions 1–9 contained no detectable envelope membranes. The sucrose density (20–38%) of fractions 15–23 is consistent with the presence of envelope membranes (53). AtPGL35 was present

FIGURE 2. Preparation of an Arabidopsis plastoglobule fraction. A, total membranes from isolated chloroplasts were separated by flotation on a discontinuous sucrose gradient. Plastoglobules are visible as a yellowish layer at the top of the gradient. Thyl, thylakoid membranes; OM/IM, outer and inner envelope membranes; PG, plastoglobules. Fractions 1 and 31 are indicated. B, SDS-PAGE analysis of fractions from the sucrose gradient. After ultracentrifugation, fractions of 1 ml were collected. Proteins contained in 400 μl of fractions 1–19, 200 μl of fractions 21–25, 100 μl of fractions 27 and 29, 25 μl of fraction 31 or 50 μl of stroma (St) were precipitated and separated by 12% SDS-PAGE, transferred to a nitrocellulose membranes, and stained with Amido Black. C, immunoblot analysis of membrane fractions using marker antibodies. The nitrocellulose membranes were probed successively with sera raised against AtPGL35, allene oxide synthase (AtAOS), tocopherol cyclase (VTE1), outer envelope membrane protein AtTOC75, inner envelope membrane protein AtTIC110, thylakoid-associated CAB, and the stromal large subunit of RuBisCO (RLSU). D, separation of proteins from low density fraction number 1 by SDS-PAGE for subsequent in-gel trypsin digestion and MS-MS analysis. Proteins contained in 1 ml of fraction 1 were precipitated by chloroform-methanol, separated on a 12% SDS-PAGE, and silver-stained.
| Name | AGL gene code | pept. nb. | Total pept. nb. | Domain prediction or and functional information | Protein length | Predictor Loc | TargetP TPloc | Present in database |
|------|---------------|-----------|----------------|-----------------------------------------------|----------------|---------------|-----------------|-------------------|
| PAP / FIBRILLINS | | | | | | | | |
| AIPGIL25 | A13g26070 | - | 4 | 4 | plastidic-lipid associated protein PAP | 242 | M | P | 50 | a, b |
| AIPGIL30 | A12g42130 | - | 5 | 5 | plastidic-lipid associated protein PAP | 270 | P | P | 48 | a |
| AIPGIL30.4 | A13g23400 | 44 | 90 | 134 | plastidic-lipid associated protein PAP | 284 | P | P | 72 | a, b |
| AIPGIL33 | A14g22240 | 28 | 38 | 66 | plastidic-lipid associated protein PAP | 310 | P | P | 59 | a, b |
| AIPGIL34 | A13g58010 | - | 33 | 33 | plastidic-lipid associated protein PAP | 308 | P | P | 53 | a |
| AIPGIL35 | A14g04020 | 52 | 108 | 160 | plastidic-lipid associated protein PAP | 318 | P | P | 55 | a, b |
| AIPGIL40 | A12g35490 | 48 | 55 | 103 | plastidic-lipid associated protein PAP | 376 | M | P | 53 | a, b |
| CHLOROPLAST METABOLISM PROTEINS | | | | | | | | |
| sugar metabolism | | | | | | | | |
| F6-BIP aldolase (FBA1) | A12g21330 | 33 | 87 | 120 | Fructose-bisphosphate aldolase class-I active site | 399 | P | P | 10 | b |
| F6-BIP aldolase (FBA2) | A14g38970 | 16 | 77 | 93 | Fructose-bisphosphate aldolase class-I active site | 398 | P | P | 46 | b |
| putative F6-BIP aldolase (FBA3) | A12g01140 | - | 5 | 5 | Fructose-bisphosphate aldolase class-I active site | 391 | P | P | 40 | b |
| jasmonic acid biosynthesis | | | | | | | | |
| allene oxide synthase (AOS) | A14g26450 | 16 | 47 | 63 | neoxanthin cleavage enzyme | 518 | M | P | 32 | a, b |
| UNCLASSIFIED PROTEINS | | | | | | | | |
| NADH dehydrogenase-like protein | A15g08740 | 3 | 30 | 33 | glutathione reductase, Dihydrolipoamide dehydrogenase, FAD-dependent pyridine nucleotide-disulfide oxidoreductase | 519 | P | P | 52 | |
| unknown | A15g05200 | 4 | 15 | 19 | ABC1 modifiable | 540 | M | M | 58 | |
| expressed protein | A17g06600 | 2 | - | 2 | ABC1 family protein, chaperonin | 711 | P | P | 42 | |
| expressed protein | A14g54570 | - | 24 | 24 | esterase/spase/leucine esterase | 704 | N | P | 27 | |
| expressed protein | A12g26640 | - | 14 | 14 | esterase/spase/leucine esterase | 701 | P | P | 65 | |
| hypothetical protein | A17g81410 | - | 3 | 3 | menaquinone biosynthesis, menaquinone-related | 355 | M | M | 49 | |
| expressed protein | A12g41040 | - | 2 | 2 | menaquinone biosynthesis-related | 352 | P | P | 55 | |
| unknown | A14g22220 | 5 | 20 | 25 | 3-beta-hydroxysteroid dehydrogenase/steroidase | 296 | P | P | 57 | |
| unknown | A12g34460 | 13 | 19 | 32 | flavine reductase, steroid biosynthesis | 280 | M | M | 51 | a |
| expressed protein | A14g13200 | - | 28 | 28 | none | 185 | N | P | 56 | a |
| unknown | A13g10130 | 6 | 9 | 15 | SOUL home-binding family protein, weak similarity to home-binding protein | 309 | P | P | 70 | b |
| unknown | A11g06690 | - | 4 | 4 | aldo-keto reductase, AIC transporter family signature | 377 | N | P | 31 | a |
| peroxiredoxin Q | A13g26060 | - | 6 | 6 | none | 216 | P | P | 57 | a, b |
| expressed protein | A11g52590 | - | 2 | 2 | none | 172 | M | P | 25 | |

**THYLAKOIDS COMPONENTS**

| Name | Protein (PsaO1, OE33) | A15g66570 | - | 6 | 6 | putative protein I photosystem II oxygen-evolving complex | 332 | P | P | 29 | a, b |
|------|-----------------------|-----------|-----------|---|---|-----------------------------------------------|----------------|---|---|---|---|
| PSII-D protein | (PsaO2, OE33) | A13g50820 | - | 3 | 3 | putative protein I photosystem II oxygen-evolving complex | 331 | P | P | 28 | a, b |
| Lhcb6-protein (CP29, Lhcb4.1) | A13g01530 | - | 2 | 2 | putative chlorophyll a-b-binding protein | 290 | P | P | 40 | a, b |
| Lhca5-protein | A11g29910 | 3 | 9 | 12 | chlorophyll a-b-binding protein | 267 | P | P | 23 | a, b |
| Lhcb5-protein (CP26, Lhcb5) | A14g10340 | - | 3 | 3 | chlorophyll a-b-binding protein I-like | 290 | P | P | 48 | a, b |
| Lhca2-protein | A13g14120 | - | 2 | 2 | PSI type III chlorophyll a-binding protein | 273 | P | P | 48 | a, b |
| chlorophyll a-binding protein | A15g54980 | - | 3 | 3 | chlorophyll a-binding protein | 241 | P | P | 35 | a, b |
| chlorophyll a-binding protein | A12g50570 | - | 2 | 2 | putative chlorophyll a-binding protein | 265 | P | P | 41 | a, b |
| PSI-L protein (PsaL, PSI subunit XI) | A10g12900 | - | 2 | 2 | probable photosystem I chlorophyll a-binding protein | 219 | P | P | 50 | a, b |
| photosystem I subunit PSI-E (PaeEII) | A14g28750 | - | 2 | 2 | photosystem I reaction center subunit IV | 143 | P | P | 44 | a, b |
| PsaL protein | A11g33310 | - | 7 | 7 | photosystem I reaction core complex | 221 | P | P | 52 | a |
| ATPase delta subunit (AbdO) | A14g06950 | - | 5 | 5 | putative ATPase | 234 | P | P | 48 | a, b |
| PSI-D protein | A14g02770 | - | 2 | 2 | photosystem I reaction center subunit II | 208 | P | P | 44 | a, b |
| ATP synthase CFI beta chain | A13g00480 | 10 | - | 10 | stpA73 (beta subunit) | 498 | - | - | - | a, b |
| psaC PSI P700 apoprotein A1 | A14g00350 | - | 3 | 3 | psaA protein: chlorophyll a-b chlorophyll a-b chlorophyll a-b | 750 | - | - | - | a, b |
| psbC PSI 43 KDa | A14g00280 | - | 4 | 4 | CP43 subunit of the photosystem II reaction center | 473 | - | - | - | a, b |

**TABLE 1**

Tandem mass spectrometry identification of proteins in the plastoglobule fraction

Proteins in the plastoglobule-containing fraction 1 from the chloroplast membrane fractionation experiment were separated by SDS-PAGE and visualized by silver staining. The gel pieces were subjected to in-gel tryptic digestion, and the tryptic peptides were extracted and subjected to tandem mass spectrometry. MS-MS data were interpreted using the SEQUEST software, allowing protein identification. Peptides were taken in account only if the corresponding protein was identified by two different peptides with scores exceeding an Xcorr value of 2.5 and a DCn value of 0.1. Proteins were divided into four groups: PAP/fibrillins, chloroplast metabolic proteins, unclassified proteins, and known thylakoid proteins. Subcellular localizations of the identified proteins were predicted by the Predotar and TargetP software. Previous identification in chloroplast proteomics projects is indicated by the presence in the plastid protein database PPDB (cbsusrv01.tc.cornell.edu/users/ppdb (58)) or in PL Prot Database (www.plprok. biol.ethz.ch/index.php/toc=91 (59)) (a).
mostly in fractions 1–11, separated from the CAB, AtTIC110, and AtTOC75 peak fractions, indicating the isolation of plastoglobules. Moreover, the sucrose density (5 and 15%) of fractions 1–11 as well as the creamy-turbid appearance of fractions at the top of the gradient (Fig. 2A) are consistent with plastoglobules (3, 12, 13, 54). Notably, separate peaks of the plastoglobule marker protein AtPLG35 in fractions 1–5 and 9–11 (5 and 15% sucrose steps) suggest the existence of plastoglobule populations of different densities, as previously reported (13).

Mass Spectrometric Identification of Plastoglobule Candidate Proteins—To identify proteins associated with plastoglobules, the low density fraction 1 (5% sucrose step) was separated by SDS-PAGE (Fig. 2D) and subjected to in-gel trypsin digestion. Tryptic peptides were extracted and analyzed by tandem mass spectrometry. The results of two replicate experiments are presented in Table 1. Protein identification was accepted if two different peptides exceeded an Xcorr value of 2.5 and a Dcn value of 0.1. Only under this condition were the peptides counted, giving a total of peptides for each identified protein. 43 proteins were identified, 40 of which are nuclear encoded. All but two of the 40 proteins are predicted by the targetP software to have a transit sequence and to be targeted to chloroplasts. The exceptions are two unknown proteins (At5g05200, At1g79600) predicted (but not known) to be mitochondrial. In the first of the two experiments, fewer proteins were identified (17 versus 41 proteins), likely due to somewhat less total protein contained in the preparation. 28 proteins were only found in one experiment. 16 of those 28 proteins, generally represented by very few peptides, were known thylakoid proteins and were, therefore, not considered to be plastoglobule components. The large subunit of RuBisCO, however, was detected neither by MS nor by immunoblot analysis (Fig. 2C), suggesting that the plastoglobule preparations were free of stroma. Fourteen proteins were present in both preparations, were not known thylakoid components, and were, therefore, good candidates for plastoglobule proteins. This number of putative Arabidopsis plastoglobule proteins is in good agreement with earlier estimations for pea plastoglobules (3) and with the number of bands visible on the SDS-PAGE gel (Fig. 2D). The plastoglobule candidate proteins fell into three categories (Table 1); PAP/fibrillin-like proteins, chloroplast metabolic enzymes, and unclassified proteins. We unequivocally identified 7 of 13 members of the Arabidopsis PAP/fibrillin family by specific peptides.

We found five known proteins involved in chloroplast metabolic pathways; two chloroplastic fructose-bisphosphate aldolase isofoms (AtFBA1 and -2, At2g21330 and At4g38970, respectively; Calvin cycle), allene oxide synthase (AtAOS, At5g42650; jasmonic acid biosynthesis (55)), a neoxanthine cleavage protein (At4g19170; abscisic acid metabolism), and VTE1 (At4g32770; tocopherol synthesis). With the exception of the two fructose-bisphosphate aldolase isofoms, the proteins are involved in pathways of plastid lipid metabolism.

Other Proteins—We identified 12 proteins annotated in the MIPS data base as “unknown.” Based on sequence similarities with known proteins, most these proteins have annotated enzymatic or metabolic functions (Table 1). Interestingly, three pairs of homologs were identified that potentially have a role in either prenylquinone synthesis (At5g05200/At1g79600, ABC1 family proteins; distantly related to ubiquinone biosynthesis protein), methyl transfer (At1g78140/At2g10140), or triacylglycerol synthesis (At1g54570/At3g26840, related to diacylglycerol acyltransferase).
**Members of the Arabidopsis PAP/Fibrillin Family Associate with Plastoglobules in Vivo**—We engineered C-terminal GFP fusion constructs under the control of the constitutive cauliflower mosaic virus 35 S promoter for three different identified PAP/fibrillins (AtPGL35, -34, and -30.4) to determine their in vivo localization. The constructs were transiently expressed in protoplasts and subsequently analyzed by confocal fluorescence microscopy (Fig. 3). GFP, FDH-GFP (presequence of potato formate dehydrogenase-GFP), and pSSU-GFP (transit peptide of small subunit of RuBisCO-GFP) were used as cytosolic, mitochondrial, and chloroplast stroma markers, respectively. To control the integrity of each GFP fusion protein, transformed protoplasts were analyzed by Western blotting using an anti-GFP serum (Fig. 3). All GFP fusion proteins had the expected mass. The three PAP/fibrillin-GFP fusion proteins (AtPGL30.4-GFP, AtPGL34-GFP, and AtPGL35-GFP) gave chloroplast fluorescence, confirming the chloroplast targeting of these proteins. Moreover, each of the PGL-GFP fusions gave globular fluorescent patterns similar to that observed in anti-AtPGL35 immunofluorescence, suggesting plastoglobule localization. Occasionally, a small fraction of the GFP fusion proteins was detected in the stroma or in the cytosol, suggesting incomplete chloroplast import or assembly into plastoglobules. None of the fusion proteins localized to the thylakoids.

**Allene Oxide Synthase and Fructose-bisphosphate Aldolases Associate with Plastoglobules**—The allene oxide synthase (AtAOS) and two fructose-bisphosphate aldolase isoforms (AtFBA1 and -2) were considered plastoglobule candidate proteins based on the large number of peptides obtained (Table 1). Upon transient expression in isolated protoplasts, AtAOS-GFP fluorescence was present in globular chloroplastic structures, suggesting association with plastoglobules (Fig. 3). The plastoglobule localization was also analyzed by immunoblotting of the fractions from the sucrose gradient using a serum raised against AtAOS (Fig. 2C). AtAOS was present in most of the fractions as well as in fractions 1 and 3, highly enriched in plastoglobules. The localization of AtAOS is, therefore, ambiguous and suggests the protein may associate with most of the chloroplast membranes.

Two isoforms of chloroplast fructose-bisphosphate aldolase were identified in the plastoglobule fraction, whereas the presence of the third was ambiguous. Although earlier work suggested that FBA is either entirely stromal or partially associated with thylakoid membranes (56), both AtFBA1-GFP and AtFBA2-GFP gave globular fluorescence patterns in chloroplasts (Fig. 3). Moreover, when AtFBA1 and AtPGL35 were co-expressed as YFP and CFP fusion proteins, overlap of the fluorescence signals indicated colocalization of the proteins in globular structures (Supplemental Fig. 1). Strong AtFBA1-GFP and somewhat weaker AtFBA2-GFP fluorescence was observed in the stroma (Fig. 3). These data suggest that both aldolase isoforms partition between the stroma and plastoglobules. Neither AtFBA1-GFP nor AtFBA2-GFP appeared to localize to the thylakoids since GFP fluorescence did not overlap with chlorophyll autofluorescence.

**Localization of Tocopherol Cyclase VTE1 in Plastoglobules**—We detected the tocopherol cyclase (AtVTE1, At4g32770) in the plastoglobule-containing fraction 1 using MS-MS. To analyze the distribution of AtVTE1 in chloroplast membranes, fractions of the sucrose gradient were probed with anti-VTE1 serum (Fig. 2C). AtVTE1 was detected in fractions 1–15 of the gradient and co-distributed with AtPGL35.

To obtain additional evidence for the localization of VTE1, ultrathin sections from 1-week-old *Arabidopsis* cotyledons were incubated with anti-VTE1 serum. As shown in Fig. 4C, plastoglobules were decorated with gold particles. However, no labeling of thylakoid or envelope membranes was observed.

Furthermore, when VTE1-YFP was expressed in protoplasts, the YFP fluorescence was present in chloroplast globules, but none was detected in either the thylakoid or the envelope membranes (Fig. 3). To determine whether VTE1 colocalized with AtPGL35 (as suggested by the membrane fractionation experiment; Fig. 2C), the two proteins were co-expressed as YFP and CFP fusion proteins, respectively, in protoplasts. AtPGL35-CFP as well as AtVTE1-YFP fluorescence was present in globular structures inside chloroplasts (Fig. 4A). Both the merge of the independent fluorescence images and the pixel intensity analysis showed an overlap of the fluorescence, indicating co-localization of AtPGL35 and AtVTE1 in plastoglobules. Taken together, the data suggest that VTE1 is neither a component of the thylakoids nor of the inner envelope membrane but is associated with plastoglobules.

**Accumulation and Enrichment of Tocopherols in Arabidopsis Plastoglobules**—Earlier studies have shown the presence of tocopherols in plastoglobules of a number of different species, but the tocopherol content of *Arabidopsis* plastoglobules and the relative abundance in membrane fractions has not yet been determined. We, therefore, determined the contents of tocopherol, fatty acids, and complex lipids of pooled fractions of the sucrose gradient, corresponding to plastoglobules, “in between,” and envelopes/thylakoid fractions. As previously described, plastoglobules were found to be rich in nonpolar lipids (triaclyglycerols, esters) but contained only low amounts of galactolipids, which make up the major fraction of thylakoid and envelope lipids (data not shown). The molar ratio of tocopherol to total fatty acid was by far the highest in plastoglobules. Fig. 5 shows that plastoglobules contain about 10 molecules of fatty acid per molecule of tocopherol, whereas in thylakoids this ratio is increased to ~250 fatty acids per molecule of tocopherol. In the different chloroplast fractions α-tocopherol was by far the most abundant form of tocopherol (data not shown). This is in good agreement with the finding that α-tocopherol is the predominant form in *Arabidopsis* leaves (57). Furthermore, the amounts of tocopherol in the different chloroplast compartments obtained from the sucrose density gradient were determined (Fig. 5B). About 36% of the total amount of tocopherol isolated from the sucrose gradient was in the plastoglobule containing fractions 1–4. Another 25% of total tocopherol was found in fractions 5–13, which are characterized by the presence of the plastoglobule marker protein AtPGL35 as well as traces of outer envelope and thylakoid markers (Fig. 2C). The data indicate that plastoglobules are an important site of vitamin E accumulation in chloroplasts.

**DISCUSSION**

We have applied a proteomic approach to determine plastoglobule functions. We used the presence of a protein in two replicate experiments as an indicator for possible plastoglobule association. The proteins identified by MS/MS fell in three major categories (Table 1): PAP/fibrillins, chloroplast metabolic enzymes, and unclassified proteins. In addition, we detected known thylakoid proteins. These are most probably due to the minor presence of the highly abundant thylakoid membranes, as suggested by the detection of a weak CAB signal in the plastoglobule-containing fractions of the gradient (Fig. 2C). Because plastoglobules have been shown to attach to thylakoid membranes (3), it is also possible that small patches of thylakoid membranes remained associated with plastoglobules during the preparation.

**PAP/Fibrillins**—Members of the PAP/fibrillin family have been known to associate with plastoglobules. It is, therefore, not surprising that we find seven of these proteins in the plastoglobule fraction. PGL30.4, -33, -35, and -40 were present in replicate plastoglobule preparations, each represented by many peptides. GFP fusions to PGL30.4, -34, and -35 were apparently targeted to plastoglobules in...
vivo (Fig. 3), ruling out their artifactual association with plastoglobules during the preparation. PGL30.4, -33, -35, and -40 have previously been shown to behave as peripheral components of thylakoid membranes by TX-114 extraction (58). In a separate study, PGL30.4 and -35 were identified in the 8M urea extract of chloroplast membranes, also suggesting a peripheral membrane association (59). It appears likely that PAP/fibrillins extracted by TX-114 or 8M urea were present in plastoglobules attached to thylakoids and, therefore, identified as thylakoid proteins.

Chloroplast Metabolic Enzymes—Among the proteins identified in the replicate plastoglobule preparations were five known metabolic enzymes (two isoforms of fructose-bisphosphate aldolases, allene oxide synthase, VTE1, and an isoform of 9-cis-epoxycarotenoid dioxygenase). The most abundant peptides belonged to two chloroplastic fructose-bisphosphate aldolase homologs (FBA1, FBA2), mostly thought to be exclusively stromal proteins. Western blotting indicated that the plastoglobule fraction did not contain a commonly used stromal marker (large subunit of RuBisCO), ruling out a simple contamination by stromal proteins (Fig. 2C). Aldolase activity, however, has previously been detected both in the stroma and thylakoid membrane preparations (56). A possible explanation to reconcile these findings is that a fraction of the aldolase associates with plastoglobules attached to the thylakoid surface, whereas, the majority of the protein is in the stroma. The presence of aldolase in plastoglobules is also supported by the mass spectrometric analysis of pea plastoglobules yielding abundant aldolase peptides, by activity measurements (data not shown), and by the colocalization of AtFBA2-YFP and AtPGL35-CFP protein fusions in cotransformed protoplasts (Supplemental Fig. 1). However, the function of aldolase in plastoglobules escapes our knowledge.

Tomato AOS has previously been described by in vitro import experiments to be targeted to the chloroplast inner envelope membrane, whereas its association with plastoglobules was not determined (60). Because we also detected AOS in envelope membrane fractions by Western blotting, we do not rule out a dual localization. However, considering that plastoglobules in chloroplasts are in tight contact with

FIGURE 4. AtVTE1 suborganellar localization. A, colocalization of AtPGL35 and AtVTE1 in protoplasts. Protoplasts were cotransformed either with AtVTE1-YFP and AtPGL35-CFP constructs or with CFP and AtPGL35-YFP constructs in a control experiment. CFP fluorescence (CFP) and YFP fluorescence (YFP) were monitored using distinct excitation wavelengths and detection windows. Pixel intensities across narrow sections, indicated by white lines on merged fluorescence images (merge), were measured. Yellow, blue, and pink pseudocolours were assigned to YFP, CFP, and chlorophyll, respectively. B, Western blot analysis of cotransformed protoplasts. Lane 1 contains proteins of protoplasts cotransformed with AtVTE1-YFP and AtPGL35-CFP constructs. Lane 2 contains proteins of protoplasts cotransformed with the CFP and AtPGL35-YFP constructs. The blot was probed with antibodies raised against GFP. The positions of the fluorescent protein fusions are indicated. C, gold immunolocalization of AtVTE1 in cotyledons. p, plastoglobules; g, thylakoid grana.
thylakoid membranes, plastoglobule localization of AOS would put the enzyme in close proximity to its oxidized lipid substrates, thought to originate from the thylakoid membrane.

We identified one of the nine Arabidopsis 9-cis-epoxycarotenoid dioxygenase (NCED) isoforms (At4g19170). NCED catalyzes the 9-cis-epoxycarotenoid cleavage reaction, a key step of abscisic acid biosynthesis, in response to water stress (61–64). The NCED isoform identified in the plastoglobule fraction, termed AtCDD4/AtNCED4 (65), has not yet been characterized nor do we provide further experimental evidence for its plastoglobule localization. However, the presence of this enzyme in plastoglobules is in good correlation with the involvement of plastoglobules in water stress responses (33) and with the presence of isoprenoid esters in plastoglobules (11, 13).

FIGURE 5. Tocopherol analysis. Total tocopherol was isolated from pooled fractions of a sucrose gradient used to separate chloroplast membranes. Tocopherol was measured by fluorescence HPLC and total fatty acids were measured by gas chromatography of fatty acid methyl esters. The data represent the means of three replicate measurements derived from a representative gradient. The numbers indicate fraction numbers (see Fig. 2). A, molar ratio of tocopherol relative to total fatty acids in the different membrane fractions. B, tocopherol distribution in the pooled fractions of the sucrose gradient. PG, plastoglobules; OM/IM, outer/inner envelope membrane; Thyl., thylakoid; Env., envelope.

A large number of peptides was attributed to AtVTE1 encoding tocopherol cyclase (66), a key enzyme in tocopherol synthesis. The Arabidopsis VTE1 protein is an ortholog of the maize sucrose export-deficient 1 protein (SXD1), previously identified by Provencher et al. (67). SXD1 was implicated in transferring chloroplast signals relevant to the carbohydrate status to the nucleus. However, SXD1 from maize was shown to encode a protein with tocopherol cyclase activity and, thus, to be a functional ortholog of Arabidopsis VTE1 (66, 68). Western blot experiments demonstrated that SXD1 from maize localizes to chloroplasts. Furthermore, incubation of radiolabeled SXD1 protein with pea chloroplasts showed that SXD1 is imported into chloroplasts and protected from protease treatment (67). These data are in agreement with the localization of SXD1/VTE1 to the chloroplast plastoglobule fraction shown in this study. Most of the enzyme activities involved in tocopherol synthesis have previously been localized to the inner envelope membrane (18). The tocopherol cyclase activity, however, was at the detection limit, suggesting that the cyclase reaction takes place in another chloroplast compartment. We present immunoelectron microscopy, Western blotting of membrane fractions, and transient expression of a YFP fusion protein as evidence for the plastoglobule localization of VTE1. The exact codistribution of VTE1 with PG35 in the membrane fractionation experiment (Fig. 2) and microscopic colocalization of corresponding YFP and CFP fusion proteins (Fig. 4) suggest that plastoglobules are the unique site of VTE1 and, therefore, tocopherol cyclase activity. Furthermore, the data suggest that VTE1 localizes to plastoglobules throughout the Arabidopsis life cycle, as it was detected in plastoglobules of cotyledons of 1-week-old plants (Fig. 4C) as well as of mature rosette leaves (Fig. 2).

The methyltransferase VTE3 involved in plastoquinone synthesis and in the synthesis of the tocopherol cyclase substrate, 2,3-dimethyl-5-phytyl-1,4-hydroquinol, was recently identified as a 37-kDa inner chloroplast envelope protein (69–71). These findings suggest that the tocopherol cyclase substrate, 2,3-dimethyl-5-phytyl-1,4-hydroquinol, is directly or indirectly transferred from the inner envelope membrane to plastoglobules where the cyclase reaction takes place. Finally, γ-tocopherol methyl transferase (VTE4) completes the synthesis of α-tocopherol, the most abundant form of tocopherol in the chloroplast. γ-Tocopherol methyl transferase was not detected in plastoglobules, suggesting that the final methylation step does not take place in plastoglobules. Plastoglobules may, therefore, not only be the site of the tocopherol cyclase but may also be involved in the trafficking of tocopherol biosynthetic intermediates. The presence of tocopherol cyclase in the plastoglobule fraction is consistent with the reported presence of tocopherols in plastoglobules (11, 13, 15). In Arabidopsis plastoglobules, tocopherols are highly enriched with regard to total fatty acid content (Fig. 5A). Moreover, Arabidopsis plastoglobules contain greater than 36% of total chloroplast tocopherol and are, therefore, identified as a substantial site of tocopherol accumulation in chloroplasts (Fig. 5B). The final destination of tocopherol is most likely the thylakoid membrane, where it has been shown to protect the photosystem II and to prevent the oxidative degradation of fatty acids by scavenging reactive oxygen species (72, 73). Under high light stress conditions, levels of tocopherols increase (72), and VTE1 is up-regulated (45). The presence of VTE1 and tocopherols (this paper) together with the reported increase in size and number of plastoglobules (33–35) provides a molecular explanation for the role of plastoglobules under conditions resulting in oxidative stress.

In conclusion, the results reported in this manuscript point to unexpected metabolic functions of the plastoglobule, indicating that plastoglobules are not mere lipid storage droplets. The evidence indicates a role of plastoglobules in tocopherol cyclase reaction of vitamin E synthesis. The presence of other enzymes suggest the involvement of plas-
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toglobules in additional metabolic pathways, the exact nature of which remains to be determined.

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