VIPP1, a nuclear gene of *Arabidopsis thaliana* essential for thylakoid membrane formation

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The conversion of light to chemical energy by the process of photosynthesis is localized to the thylakoid membrane network in plant chloroplasts. Although several pathways have been described that target proteins into and across the thylakoids, little is known about the origin of this membrane system or how the lipid backbone of the thylakoids is transported and fused with the target membrane. Thylakoid biogenesis and maintenance seem to involve the flow of membrane elements via vesicular transport. Here we show by mutational analysis that deletion of a single gene called *VIPP1* (vesicle-inducing protein in plastids 1) is deleterious to thylakoid membrane formation. Although VIPP1 is a hydrophilic protein it is found in both the inner envelope and the thylakoid membranes. In *VIPP1* deletion mutants vesicle formation is abolished. We propose that *VIPP1* is essential for the maintenance of thylakoids by a transport pathway not previously recognized.

The biogenesis of thylakoid membranes of higher plants requires the coordinated synthesis of proteins in the plastidial and the cytosolic compartment (1). The proteins of cytosolic origin have to be transported into the plastids, targeted to their intraorganellar destination, and then assembled with their plastid-encoded counterparts into multisubunit complexes that are typical for this photosynthetic membrane (reviewed in refs. 2–4). Most of the thylakoid membrane complexes contain nonproteinaceous cofactors such as pigments or metal ions. The assembly process is, therefore, also dependent upon the on-time delivery of these factors (4).

The photosynthetic protein complexes are embedded in a lipid matrix that is about 70–80% galactolipids (5). The galactolipids are synthesized at the inner envelope membrane (6), and therefore intraorganellar lipid transport systems must exist that ensure the transfer of lipids from their site of synthesis to the thylakoids.

In developing (7) but also in mature (8) chloroplasts of higher plants and green algae, infolding of the inner membrane has been observed, suggesting that transient fusions between the inner envelope and thylakoid membranes provide a means for lipid transfer. However, lipids could also be transferred from the inner envelope to the thylakoids by specific transfer proteins or by some sort of vesicular transport system (9). Indeed, there is growing evidence that the vesiculation of the envelope inner membrane provides the pathway by which lipids are transported from the site of synthesis at the inner envelope to the thylakoids. The budding of vesicles from the inner envelope has been documented by several electron microscopic studies (7, 10, 11), and tentative evidence indicates that these vesicles may fuse with each other (12) or with growing thylakoids (10). Formation of vesicles is more intense when plants are subjected to various environmental conditions, including incubation at lower temperatures (11).

Biochemical and molecular biological studies support the existence of a vesicular transport system in chloroplasts. These studies succeeded in isolating an NSF-like protein from pepper chromoplasts (12) and a dynamin-like protein from *Arabidopsis* chloroplasts (13), respectively. Both proteins are well known components of cytosolic vesicular transport systems (reviewed in refs. 14 and 15). Thus all available cytological and biochemical data indicate that plastids contain such a transport system. However, nothing is known about the cargo this system transports and how the transport machinery is organized.

Here we report on a pleiotropic high-chlorophyll fluorescence mutant of *Arabidopsis thaliana* (*hcf155*) that provides a genetic entry point for the analysis of thylakoid formation. The mutant is deficient in vesicle budding from the inner envelope membrane. The lack of vesicle formation is paralleled by the inhibition of thylakoid formation. The mutated gene of *hcf155*, named *VIPP1* (vesicle-inducing protein in plastids 1), was cloned by T-DNA tagging.

Materials and Methods

Isolation of Mutant Plants and Their Spectroscopic and Biochemical Characterization. The mutant *hcf155* (originally named COD21) was isolated from a collection of T-DNA *Arabidopsis* lines that were produced by the Institut National de la Recherche Agronomique Laboratoire de Génétique et Amélioration des Plantes at Versailles (17, 18). Mutant plants exhibiting the phenotype of high chlorophyll fluorescence were selected in the dark under UV light as described (19). Mutant plants were grown on sucrose-supplemented Gelrite medium (19) at 23°C and a photon flux density of white light of 20–50 $\mu mol \cdot m^{-2} \cdot s^{-1}$ with a 16-h photoperiod. Chlorophyll fluorescence measurements, P700 redox kinetics, immunological analyses of thylakoid membrane proteins, and Northern hybridizations were carried out as described (19), with the exception that for immunoblot analyses of thylakoid membrane proteins total leaf extracts were used.

Amplification of T-DNA-Flanking Genomic Sequences. Genomic sequences flanking the T-DNA right border were amplified by inverse PCR (20). Genomic DNA of *hcf155* digested with *Eco*RV was self-ligated, and the genomic sequences were amplified by touchdown PCR. Annealing started at a temperature of 63°C and was then decreased in steps of 0.5°C to a final temperature of 55°C. A concentration of 20 μM of each T-DNA-specific primer (5′-GGACTGACCACCCCAAGTGC-3′ and 5′-CAGGGCGGCTATACGCCATT-3′) was used for amplification. With the exception of the touchdown cycles, the PCR was carried out according to standard protocols (21). The obtained DNA fragment of 1.3 kb was cloned (clone pnAt155-RB3) and sequenced. A 1189-bp fragment of the AAC27134 gene was

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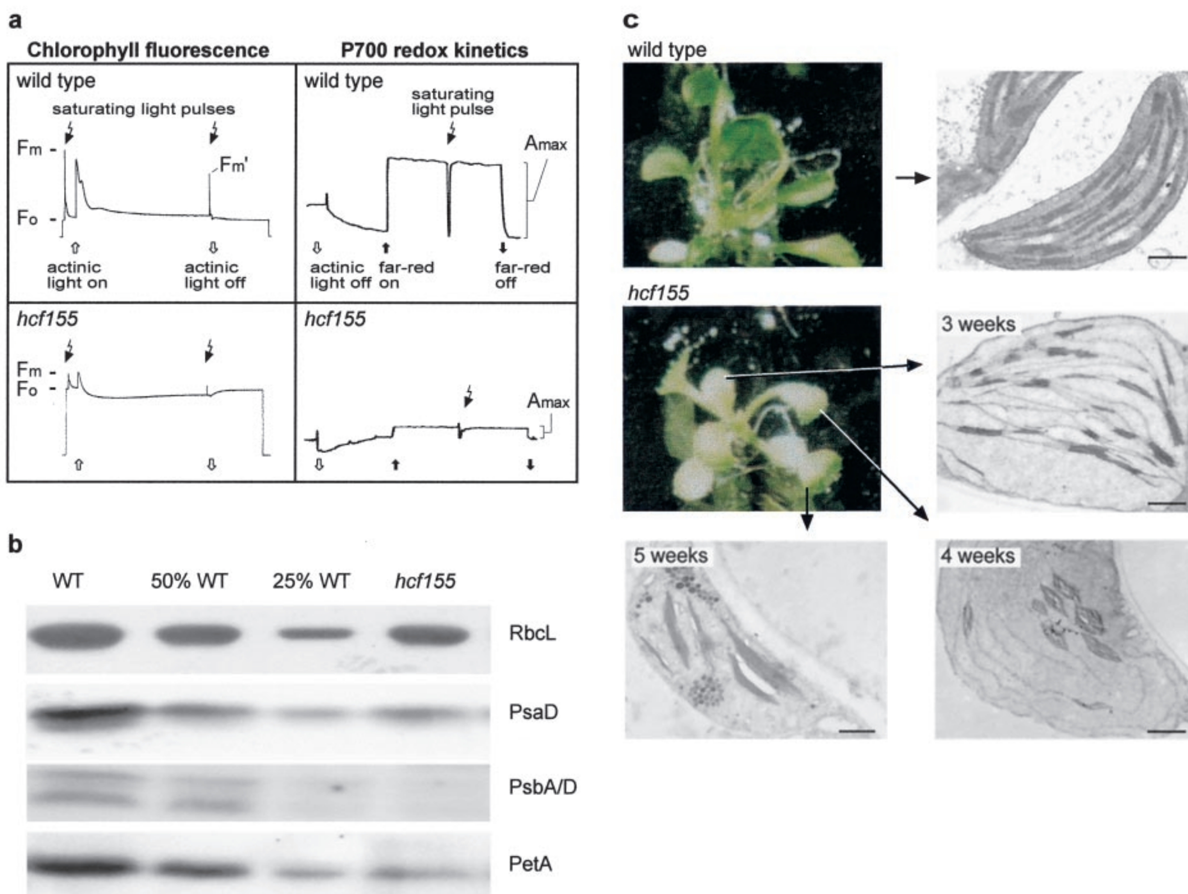

Fig. 1. The high chlorophyll fluorescence mutant *hcf155* is deficient in function and structural integrity of thylakoid membranes. (*a*) Measurements of chlorophyll fluorescence induction and P700 redox kinetics of *hcf155* mutant and wild-type plants of *Arabidopsis thaliana*. The minimal level of fluorescence (Fo) of dark-adapted whole plants was measured by switching on a pulsed measuring beam of red light. A saturating pulse of white light was then applied to determine the maximum level of fluorescence in the dark-adapted state (Fm). Subsequently actinic (white) light was switched on to drive photosynthesis. A further saturating flash allowed us to measure the fluorescence maximum in the light (Fm′). The activity of photosystem I was investigated by measuring absorbance changes of P700 at 830 nm (A_{max}) induced by far-red light (720 nm). Plants were preilluminated for 4 min with actinic light. Far-red light was switched on to oxidize P700. A saturating pulse of white light was then applied to rereduce P700 and thus to determine A_{max}. (*b*) Immunoblot analysis of representative photosynthetic proteins in *hcf155* mutant and wild-type (WT) plants. Total membrane proteins were isolated from mutant and wild-type plants, separated on SDS/polyacrylamide gels, and transferred to nitrocellulose membranes. Immunoblots were performed with antisera made against RbcL, the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase; PsaD, subunit D of photosystem I; PsbA/D, subunits D1 and D2 of photosystem II; and PetA, cytochrome *f*. Fifteen micrograms of total protein (WT and *hcf155*) or, in the case of the wild-type extract, corresponding dilutions were analyzed. (*c*) Electron micrographs showing the ultrastructure of chloroplasts from mutant leaves of progressing age. (*Top*) An electron micrograph of a typical wild-type chloroplast. (Scale bars represent 1 μm.)

amplified by using the available sequence information of that gene (primers: 5′-CTCTCAAAGCTTCACCTGTTACCG-3′ and 5′-CTCACGTGCAAGATCCTCATCTCC-3′).

Cloning of *VIPP1* cDNA and Mutant Complementation. Single-stranded cDNA was synthesized from total *hcf155* RNA with the use of oligo(dT) primers and avian myeloblastosis virus reverse transcriptase (Roche Molecular Biochemicals), following the manufacturer's instructions. The *VIPP1* coding sequence was amplified according to standard protocols (21) with the use of the 5′ *VIPP1* primer 5′-CGCCATTGAAATGGCTCT-CAAAGCTTCACC-3′ and the 3′ *VIPP1* primer 5′-GTAAC-CCAGTCACAATTCGGAAACAGCCG-3′. The amplified DNA was cloned, and several of the obtained clones were fully sequenced. One of the clones with a correct *VIPP1* sequence named pcVIPP1 was used for the complementation of homozygous *hcf155* plants essentially as described (22, 23). More than 20 independent transgenic plants were recovered.

Immunolocalization of the *VIPP1* Protein. Intact chloroplasts were isolated from 3- to 4-week-old *Arabidopsis* wild-type plants by Percoll gradient centrifugation (23). Chloroplasts were ruptured under hypertonic conditions with a Dounce homogenizer, and the plastidial membrane systems were separated by sucrose density gradient centrifugation (24). Immunoblot analysis of the protein fractions was carried out according to standard procedures (19).

Transmission Electron Microscopy. Leaves of wild-type and mutant plants were cut into 1-mm pieces and incubated for 45 min in water at room temperature or at 4°C. They were incubated for 5 h at 4°C in 2% glutaraldehyde and 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.2). The fixed leave pieces were washed three times for 20 min in 0.1 M sodium phosphate buffer (pH 7.2) at 4°C and then contrasted by incubation in 2% OsO_4 overnight at 4°C. After washing in 0.1 M sodium phosphate buffer (pH 7.2), samples were dehydrated in a graded ethanol series and embedded in LR White resin according to standard procedures. Ultrathin section were cut with a diamond knife and put on single-hole grids covered with a Formvar film. They were stained by incubating for 10 min in 2% aqueous uranyl acetate

followed by 5 min of incubation in lead citrate. The sections were viewed under a Philips CM10 electron microscope.

Results

Hcf155 Is Deficient in Photosynthesis. Homozygous *hcf155* mutants were unable to grow photoautotrophically on soil but could be maintained by cultivation on sucrose-supplemented agar media. The mutant plants were comparable in size to wild type but were pale green. To determine whether the mutant phenotype was due to a defect in photosynthesis, the function of the photosynthetic electron transport chain was analyzed in intact plants by spectroscopic techniques (19). Chlorophyll fluorescence induction measurements allowed us to probe the functional state of photosystem II, as it may be affected by processes occurring in the photosystem itself but also in the subsequent photosynthetic electron transport chain, i.e., the cytochrome b_6/f complex and photosystem I. The functional state of the latter was assessed by measuring the redox kinetics of P700, the reaction center chlorophyll of that photosystem. These investigations revealed that *hcf155* mutants are severely disturbed in photosynthetic electron transport. However, the defect could not be localized to a deficiency of a single thylakoid membrane protein complex but appeared to be caused by a dysfunction of the entire electron transport chain (Fig. 1*a*).

The pleiotropic photosynthetic phenotype of *hcf155* was confirmed by immunoblot analyses of total leaf extracts (19) with the use of antisera to representative subunits of photosystems I (PsaD) and II (PsbA/D), the cytochrome b_6/f complex (PetA), and the Calvin cycle enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (RbcL). The protein complexes constituting the photosynthetic electron transport chain, i.e., photosystems I and II and the cytochrome b_6/f complex, were reduced by more than 50% as compared with wild type. In contrast, the reduction in stromal ribulose-bisphosphate carboxylase/oxygenase levels was found to be much less severe (Fig. 1*b*).

The Thylakoid Membrane System of _hcf155_ Is Degraded. To see if the *hcf155* mutant was also affected on the ultrastructural level, electron micrographs of ultrathin sections from leaves were analyzed. Chloroplasts from young emerging 3-week-old leaves of mutant plants showed a disturbed thylakoid membrane system with widely and irregularly spaced lamellae (Fig. 1*c*). The thylakoid structure was even more distorted in older expanded leaves. Typically, in 4-week-old leaves some unstructured membranes were left. Moreover, some irregularly shaped membranous inclusions could be observed. At 5 weeks most of the unstacked thylakoids had disappeared, whereas membrane material, like proteins, chlorophylls, carotenoids, and maybe even lipids, seemed to be packed into globular or membranous structures of high electron density (Fig. 1*c* and data not shown). We conclude that the gene mutated in *hcf155* is essential for the acquisition and maintenance of structurally and functionally intact thylakoid membranes. The virtual absence of thylakoids (i.e., of photosynthesis) explains the failure of *hcf155* plants to grow photoautotrophically.

VIPP1 Is Responsible for the _hcf155_ Mutant Phenotype. Genetic analyses revealed that the mutation responsible for the *hcf155* phenotype is recessive and that the herbicide resistance marker carried by the T-DNA insertional mutagen cosegregates with the mutation. These findings suggest that the T-DNA insertion caused the mutant phenotype of *hcf155* and that the corresponding gene should therefore be tagged. To isolate this gene, genomic sequences flanking the right border of the T-DNA were amplified by inverse PCR, sequenced, and compared with available *Arabidopsis* genomic sequences. These analyses showed that the T-DNA is inserted on chromosome 1 between two putative genes with the ID numbers AAC27134 and AAC27135 (Fig. 2*a*).

Fig. 2. The T-DNA insertion in *hcf155* reduces the expression of a gene [gene designation *VIPP1* (AAC27135.1)] that encodes a chloroplast protein that is located in both the inner envelope and the thylakoid membrane. (*a*) Localization of the T-DNA insertion site and identification of the affected gene by Northern blot analysis. The hybridization probes specific for the AAC27134 and AAC27135 genes that flank the T-DNA insertion were generated by PCR. RNA loadings were 10 μg of total RNA (WT and *hcf155*) and a 50% dilution in the case of wild-type RNA. (*b*) Amino acid alignment of the VIPP1/IM30 precursor proteins of *Arabidopsis thaliana* (At) and *Pisum sativum* (Ps). The putative cleavage sites are indicated. (*c*) Localization of the VIPP1 protein in plastid fractions of *A. thaliana* wild-type plants with the use of marker proteins of established topology: Tic40, subunit of the protein translocation machinery of the inner envelope (IE) (29); Toc160, subunit of the protein translocation machinery of the outer envelope (OE) (16); RbcL, large subunit of the stromal (STR) enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase; LHCP, major chlorophyll *a*/*b*-binding antenna protein of photosystem II, a thylakoidal (THY) protein complex. Equal amounts of protein (8 μg per lane) were loaded onto the gel.

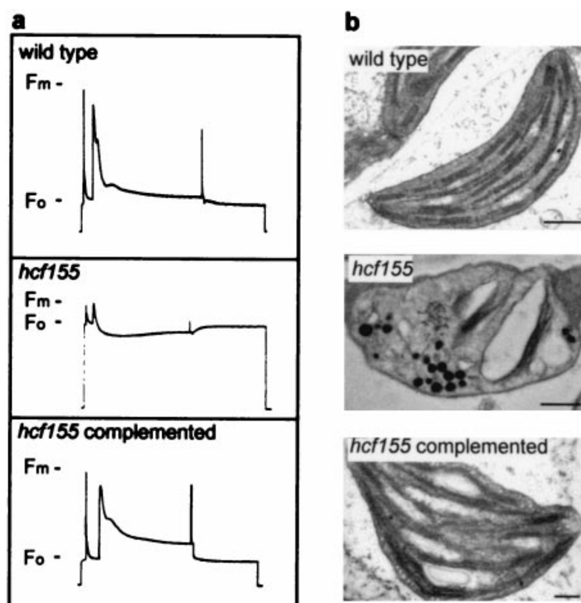

Fig. 3. A full-size cDNA encoding the VIPP1 protein of *A. thaliana* and driven by the cauliflower 35S promoter complements the *hcf155* mutant phenotype. (*a*) Chlorophyll fluorescence traces of wild-type and *hcf155* homozygous mutant plants are shown for comparison. (*b*) Electron micrographs of chloroplasts from complemented mutant plants in comparison with wild-type and homozygous *hcf155* mutant plants. (Scale bars represent 1 μm.)

To determine which of the genes is affected by the T-DNA insertion, Northern blot hybridizations (19) with gene-specific probes (pnAt155-RB3 and pnAt155-LB2) were performed. These analyses showed that the inserted T-DNA affects only the AAC27135 gene by reducing the steady-state level of the corresponding transcript to about 5–10% of that of the wild type (Fig. 2*a*). Because the T-DNA is inserted in the 5′ flanking sequence of the AAC27135 gene, the reduced RNA levels are most probably due to a decreased rate of transcription.

To prove that the reduced accumulation of AAC27135 transcripts was causing the mutant phenotype, a cDNA with the entire AAC27135 sequence was introduced into homozygous mutant plants by *Agrobacterium tumefaciens*-mediated transformation (23). This rescue resulted in *Arabidopsis* plants that were capable of photoautotrophic growth on soil and could set seeds. The chlorophyll fluorescence curves of the complemented mutant plants were close to wild type but exhibited decreased levels of photochemical (qP) and nonphotochemical (qN) quenching (Fig. 3*a*). These slight deviations from wild type are typically observed in complemented mutant plants (K.M. and P.W., unpublished data) and may indicate that the regeneration procedure causes stress to the plants. The chloroplast ultrastructure of the complemented mutant plants was indistinguishable from that of wild type (Fig. 3*b*). We conclude from these results that the *hcf155* mutant allele of AAC27135 is causing the observed mutant phenotype, and we named the identified gene *VIPP1*.

VIPP1 Encodes a Plastidial Membrane Protein of Eubacterial Origin. *VIPP1* encodes a protein of 330 amino acids whose N-terminal part exhibits features typical for plastid transit sequences. A putative cleavage site for removal of the transit peptide can be found at amino acid position 53 (VLRLA; Fig. 2*b*), predicting a mature VIPP1 protein about 32 kDa in size. VIPP1 homologues in plants were detected by BLAST searches. The closest similarity (70% identity) was observed with the IM30 protein of pea (Fig. 2*b*) (25). We detected lesser but still significant sequence similarities to two putative gene products in both *Synechocystis* PCC6803, sll0617 and slr1188 (26), and *Anabaena*, anac361a and anac361b (Kazusa DNA Research Institute), as well as to other eubacterial homologues such as the PspA protein of *Escherichia coli* (25, 27, 28). These similarities suggest that the *VIPP1* genes are of ancient phylogenetic origin.

The VIPP1/IM30 protein of pea has been described as a plastid protein of unknown function that is associated with both the inner envelope and thylakoids (25). Immunoblot experiments using an antibody to the pea protein confirmed that this

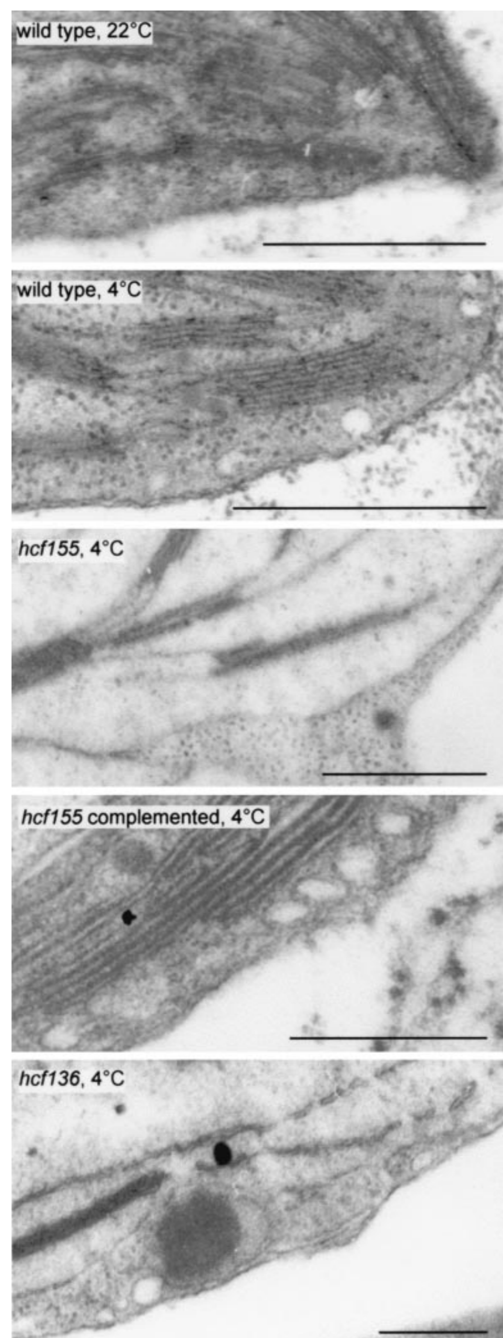

Fig. 4. *hcf155* mutant plants are deficient in the budding of vesicles from the inner envelope membrane. Representative electron micrographs are shown. The formation of vesicles can be observed in leaves from wild-type plants incubated at 4°C for 45 min, but not at 22°C. Vesicle budding is lacking in homozygous *hcf155* mutant seedlings but not in the photosystem II mutant *hcf136* (20). Complemented *hcf155* mutant regains the ability to form vesicles. (Scale bars represent 1 μm.)

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description is also true for the VIPP1 homologue of *Arabidopsis* (Fig. 2*c*). Two variants of the VIPP1 protein differing in size by about 2 kDa were detected in the thylakoid membrane fraction. In pea, where a similar effect was observed, amino acid sequencing of the two forms revealed an identical N-terminal sequence, indicating that internal modifications could be responsible for the shift in mobility (data not shown). The functional significance of these size differences remains unclear at present.

A Functional VIPP1 Protein Is Required for Plastid Vesicle Formation. Li *et al.* (25) suggested that the VIPP1/IM30 protein could be involved in transport from the envelope to the thylakoids. They speculated that the protein might function as a sort of lipid transfer protein for galactolipids or as part of a vesicular transport system. To determine whether the *hcf155* mutant is affected in the formation of vesicles, an electron microscopic analysis was performed. Because low temperatures are known to increase the accumulation of vesicles in plastids (10), this treatment was applied to *Arabidopsis*.

The electron micrographs presented in Fig. 4 show that vesicles bud from the inner envelope in the plastids of wild-type *Arabidopsis* plants when exposed to low temperatures. In contrast, no vesicles accumulate in the *hcf155* mutant (Fig. 4). In *hcf136*, a photosystem II mutant of *Arabidopsis* (23), vesicle formation was comparable to that of the wild-type plants, demonstrating that the lack of vesicle formation in *hcf155* is not a secondary effect due to a deficiency in photosynthesis. We conclude that VIPP1 is a prominent factor of a vesicular transport system required for thylakoid formation.

Discussion

In the present study we have identified a nuclear-encoded plastidial protein, VIPP1, that is involved in the budding of vesicles from the inner envelope, which in turn is required for the maintenance of a structurally and functionally intact thylakoid membrane. The protein is of eubacterial origin, suggesting that its function is conserved during evolution (see ref. 28). According to hydropathy analysis (data not shown), VIPP1 can be classified as a hydrophilic protein. However, the investigation of its distribution within the chloroplast revealed that VIPP1 is bound to both the thylakoid and the inner envelope membrane (ref. 25 and this study). It is not yet known which part of the protein binds to the membranes and what the interacting component(s) at the membrane site might be.

The phenotypic data obtained with *hcf155* can best be explained by assuming that VIPP1 is involved in a vesicular transport system that connects the inner envelope and the thylakoids. The association of VIPP1 with both the thylakoids and the inner envelope membranes is consistent with this conclusion, as are immunocytochemical data which show that VIPP1 occurs in clusters in the vicinity of both the envelope and the thylakoid (25). However, VIPP1 does not show any similarity to known proteins of the cytoplasmic vesicular transport system. Because preliminary biochemical and molecular biological studies provide tentative evidence that plastids may contain a similar system, the precise role of VIPP1 in the cycle of vesicle budding, migration, and fusion remains an enigma.

If polar lipids were a major cargo of plastid vesicles, an effect on the lipid composition of mutant plants could be imagined. So far, a comparison of the various lipid classes between 3- to 4-week-old wild-type and mutant plants did not reveal any significant differences (data not shown), indicating that VIPP1 is not required for overall lipid accumulation. Whether VIPP1 is involved in the transport of lipids from the envelope to the thylakoids remains to be seen, inasmuch as envelope and thylakoid membrane fractions could not be isolated from the mutant plants in sufficient quantities for lipid analysis. Regardless of its exact role, the importance of VIPP1 becomes even more eminent because of a parallel study (28) of cyanobacteria in which deletion of the *VIPP1* homologue in *Synechocystis* led to a complete loss of thylakoid formation. VIPP1 defines an essential component of a pathway for membrane biosynthesis that would be a worthwhile subject for further study.

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