# FUNCTIONAL TRAITS OF LIPID PARTICLES ORIGINATING FROM THYLAKOID MEMBRANES

by

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## ABSTRACT

Lipid-protein particles bearing the 55 kD Rubisco large subunit (RLSU) and no detectable corresponding Rubisco small subunit (RSSU) were isolated from the stroma of intact *Phaseolus vulgaris* chloroplasts by flotation centrifugation. Stromal RLSU-bearing particles appear to originate from thylakoids inasmuch as they can also be generated *in vitro* by illumination of isolated thylakoids. Their formation *in vitro* is facilitated by light or ATP and can be inhibited by prior heat-denaturation of the membranes. The RLSU-containing lipid-protein particles range from 0.05 to 0.10 μm in radius, contain the same fatty acids as thylakoids, but have a 10- to 15-fold higher free to esterified fatty acid ratio than thylakoids. RLSU-bearing lipid-protein particles with no detectable RSSU were also immunopurified from the populations of both stromal lipid-protein particles and those generated *in vitro* from illuminated thylakoids. Protease-shaving indicated that RLSU is embedded in the lipid-protein particles and that there is also protease-protected RLSU in thylakoids. These observations collectively indicate that RLSU associated with thylakoids is released into the stroma by light-facilitated blebbing of lipid-protein particles. The release of RLSU-containing particles may in turn be coordinated with the assembly of Rubisco holoenzyme inasmuch as chaperonin 60 is also associated with lipid-protein particles isolated from stroma.

Two subpopulations of lipid particles isolated from chloroplasts of *Phaseolus vulgaris* leaves have been found to contain PAP (plastid-lipid-associated protein). One subpopulation is comprised of plastoglobuli isolated from sonicated thylakoids by flotation centrifugation according to a standard procedure. The second subpopulation is comprised of higher density particles, previously referred to as lipid-protein particles (Ghosh et al., 1994), present in the chloroplast stroma. Since these particles contain PAP but have a higher buoyant density than

plastoglobuli, they have been termed plastoglobuli-like particles. Of particular interest is the finding that plastoglobuli and plastoglobuli-like particles also both contain catabolites of the thylakoid protein, cytochrome *f*. These observations support the view that there are distinguishable populations of plastoglobuli-like particles in chloroplasts. They further suggest that the formation of these particles may allow removal of cytochrome *f* catabolites from the thylakoid membrane that are destined for degradation as part of normal thylakoid turnover.

A lipase gene is up-regulated in the leaves of *Phaseolus vulgaris* as a function of age and in response to exogenous ethylene, as indicated by Northern blot analysis using a heterologous cDNA as a probe. A series of degenerate primers were designed based on the amino acid and nucleotide sequences of seven plant lipases, and used to amplify putative lipase clones from 3-week-old primary leaf RNA by RT-PCR. Three partial clones were obtained, all of which corresponded to lipases as determined by comparison to protein databanks. Sequence analysis indicates that one or more of the leaf lipases may correspond to a lipolytic acyl hydrolase, may be regulated by phosphorylation and could be targeted to chloroplasts. Lipolytic acyl hydrolases from *Phaseolus* leaves capable of degrading galactolipids have been described on a biochemical level, but genes for such enzymes have not been isolated.

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for everything.

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# 1. INTRODUCTION

## 1.1 PLASTIDS

Plastids are one of the characteristic features of plant cells. These organelles are not formed *de novo*, but rather differentiate from precursors that are maternally inherited. The simple precursors are termed eoplasts or proplastids, and are small, colourless organelles with very little, if any, internal structure (Wellburn, 1984; Hudak, 1997). Proplastids can differentiate into various types of plastid depending on environmental conditions and the tissue in which they reside. Leaf plastids undergo a well-characterized sequence of structural changes during development in the light to form photosynthetic chloroplasts (Wellburn, 1984; Hudak, 1997). In contrast, if allowed to develop in the dark, leaf plastids will develop into colourless etioplasts with an extensive internal membrane system that differs from thylakoids and is referred to as prolamellar bodies. Upon illumination, etioplasts can be transformed into chloroplasts. Other non-photosynthetic plastids include colourless leucoplasts, which are essentially undifferentiated proplastids, amyloplasts, which are starch-storing plastids most often found in stems, roots and tubers, and elaioplasts, which are lipid-storage plastids found in, for example, tapetal cells (Douce and Joyard, 1990). Finally, chromoplasts are colourful, carotenoid-rich plastids present in petals, fruits and roots.

Chloroplasts are complex organelles, with six suborganellar compartments, namely the outer envelope membrane, the inner envelope membrane, the intermembrane space, the stroma, the thylakoid membranes and the thylakoid lumen. All plastids also contain a small, autonomously replicating, circular, single-chromosome genome, often referred to as the plastome or cpDNA (Matile, 1992; Hachtel, 1997). The plastid genome is usually between

120 and 190 kb in size (depending on the species) and is known to code for about 100 proteins in addition to rRNAs (3-5 genes) and tRNAs (30-31 genes) (Sugiura, 1992; Hachtel, 1997). Some open reading frames (ORFs) of cpDNA that are potential polypeptide-encoding genes are still being characterized. For example, the smallest conserved open reading frame in the plastid genome, which encodes a polypeptide component of the cytochrome $b_6/f$ complex, was only recently characterized (Hager et al., 1999). This ORF, *ycf6*, was shown to encode a 29 amino acid hydrophobic polypeptide crucial for the assembly of the cytochrome $b_6/f$ complex, and was renamed *petN* (Hager et al., 1999).

Plastid-encoded proteins are produced by plastid protein expression machinery. The mechanism of plastid gene expression is somewhat different from that of the nucleus/cytosol. Specifically, chloroplastic RNA polymerase and the overall process of transcription are more similar to that of prokaryotes than eukaryotes. In addition, translation of plastid transcripts occurs in the stroma on prokaryotic-like 70S ribosomes that are distinct from the 80S ribosomes found in eukaryotic cytosol. While plastids are capable of synthesizing many of their own proteins, the majority of chloroplast proteins are encoded by the nuclear genome and synthesized as precursors in the cytosol (Chen and Schnell, 1999). These precursors contain a cleavable chloroplast-targeting signal on their N-terminus and are imported into the chloroplast by the well-characterized general import machinery localized on the chloroplast envelope membranes (Fuks and Schnell, 1997; Soll and Tien, 1998). Once imported, the protein is processed and further targeted to its proper destination within the plastid (Robinson et al., 1998; Schnell, 1998).

Chloroplasts are ovoid-shaped organelles, usually 4-8 μm in diameter, that can be present in 1 to 400 copies per leaf cell depending on the species (Kirk, 1978). It has been estimated

that a mature potato leaf contains approximately 380,000 chloroplasts per $mm^2$ of leaf (Kirk, 1978). They are the best-characterized type of plastid, and the site of photosynthesis.

## 1.2 ORGANIZATION AND BIOCHEMISTRY OF CHLOROPLASTS

### 1.2.1 Chloroplast Envelope

The plastid envelope is composed of two separate membranes, the outer and inner envelope membranes. The space between the membranes of the double envelope is referred to as the intermembrane space. The outer membrane contains non-specific pore proteins that allow free passage of water and molecules smaller than 10 kDa into the intermembrane space (Flügge and Benz, 1984; Douce and Joyard, 1990). The inner membrane acts as a more typical semi-permeable barrier to large and small compounds. Together, the envelope membranes regulate the inflow of metabolites and the outflow of photosynthetic products, as well as the translocation of nuclear-encoded chloroplast proteins from the cytosol into the plastid through the general import machinery (Fuks and Schnell, 1997; Koike et al., 1998). The envelope is also the site of galactolipid synthesis (Carde et al., 1982). Galactolipids are the most abundant polar lipids of chloroplasts and are not found anywhere else in the plant cell.

The envelope membranes are composed of the same lipids as the internal membranes of the chloroplast (thylakoids), although the lipids are present in different relative proportions. Specifically, envelope membranes contain fewer galactolipids than thylakoids, and monogalactosyl diacylglycerol (MGDG) and digalactosyl diacylglycerol (DGDG) are present in a ratio of 0.3-0.8:1, as compared to 2:1 in thylakoids (Douce and Joyard, 1980). Envelope membranes also contain more phosphatidylcholine than thylakoids, and the fatty acids of the envelope are generally more saturated than those of the photosynthetic membranes. The most

consistent and obvious biochemical difference between envelope and thylakoid membranes is the absence of chlorophyll from the envelope. Interestingly, however, enzymes involved in the biosynthesis of chlorophyll as well as chlorophyll biosynthetic precursors, are present on envelope membranes (Douce and Joyard, 1990; Hoober et al., 1994; Reinbothe and Reinbothe, 1996). As well, the carotenoid, violaxanthin, has been identified as a component of all plastid envelopes (Douce and Joyard, 1990).

### 1.2.2 Stroma

The stroma is the soluble protein matrix of plastids. In chloroplasts, it contains at least 140 different proteins, which are involved in DNA replication, transcription, translation and carbon fixation (de Boer and Weisbeek, 1991). Ribulose-1,5-bisphosphate carboxylase/ oxygenase (Rubisco) accounts for approximately 50% of the total stromal protein in $C_3$ higher plant species and catalyzes the first step in the fixation of $CO_2$ (Hattori and Margulies, 1986). In fact, the stroma contains all the enzymes of the Calvin cycle, which uses ATP and NADPH produced during photophosphorylation to convert $CO_2$ into carbohydrate (Halliwell, 1984; Hope, 1993). Many of these enzymes are found in functional multienzyme complexes, which are thought to allow channeling of intermediates between the enzymes of the pathway (Gontero et al., 1988).

### 1.2.3 Thylakoids

Thylakoids, also referred to as lamellae, form the extensive internal membrane system of chloroplasts, and are the structural feature that distinguishes chloroplasts from other types of plastid. The thylakoid membrane is arguably the most complex of all biological membranes

and is the most abundant membrane system of plants (Hoober et al., 1994). Chloroplasts contain up to 63% of total leaf lipid, and the majority (90%) of this lipid is present in the thylakoids (Kirk, 1978; Rawyler et al., 1995). This means that a single type of membrane accounts for more than half of the lipid content of a typical leaf. The primary function of thylakoids in photosynthesis is to capture light energy and convert it into chemical energy (ATP and NADPH), which is then used primarily to drive the carbon-fixing Calvin cycle in the stroma. Specifically, the thylakoids house the complexes involved in harvesting light energy and using it to transfer electrons from water to $NADP^+$, liberating $O_2$ and NADPH. In the process, a pH gradient is generated across the membrane, which is then used to drive the synthesis of ATP.

Thylakoids are sac-like membranes enclosing a soluble matrix separate from the stroma, called the lumen (Albertsson, 1995). The lumen is a densely packed, highly acidic space that has been reported to contain approximately 25 different polypeptides, not all of which have been characterized (Kieselbach et al., 1998). Some lumenal-specific proteins that have been characterized include plastocyanin, chaperonin 60, chaperonin 10, heat shock cognate protein 70, and a 40 kDa immunophilin-like protein involved in protein folding (Schlicher and Soll, 1996; Fulgosi et al., 1998; Sigfridsson, 1998). The lumen is very acidic due to the continual pumping of protons into the space from the stroma during photosynthetic electron transport (Kramer et al., 1999). The resulting proton motive force drives the synthesis of ATP by the ATP synthase complex. The lumen is also the site of water splitting and the resulting evolution of oxygen by the oxygen-evolving complex associated with photosystem II (Melis, 1991; Wollman et al., 1999).

The membranes of the thylakoids form two functionally and physically distinct domains, the grana and stroma lamellae (Albertsson, 1995). The grana are formed by stacking of thylakoid sacs, and can be further sub-divided into appressed and non-appressed regions as well as the granal margins. Appressed membranes are those that are in close contact with other membranes of the grana and constitute the core of the membrane stack. The membranes in this domain of the grana are not exposed to stroma. In contrast, the margins constitute the peripheral domains and the non-appressed regions constitute the end membranes of the granal stacks (Albertsson, 1995). Both of these latter domains are exposed to stroma. The stroma lamellae are continuous with the grana and often link grana together, but exist as single sheets fully exposed to stroma rather than as stacked membranes. The physically distinct domains are also heterogeneous at the molecular level, in that they have distinct protein compositions. For example, stroma lamellae are enriched in Photosystem I (PS I) and the ATP synthase complex, whereas the grana lamellae are enriched in Photosystem II (PS II). The cytochrome $b_6/f$ complex is evenly distributed between the two domains (Olive et al., 1986; Albertsson, 1995).

#### *1.2.3.1 Thylakoid Lipids*

The major lipids of the thylakoid membrane are monogalactosyl diacylglycerol (MGDG), digalactosyl diacylglycerol (DGDG), sulfoquinovosyl diacylglycerol (SQDG) and negatively charged phosphatidylglycerol (PG). MGDG and DGDG are present in a ratio of about 2-to-1, and together account for 80% of the thylakoid lipids. The stoichiometry of the four major glycerolipids is an essential factor in maintaining the structure and stability of thylakoids (Quinn and Williams, 1983). The galactolipids are enriched in highly unsaturated fatty acids (especially linolenic acid, $C_{18:3}$), making thylakoids very fluid membranes (Douce and Joyard,

1980; El Maannii et al., 1998). For example, it has been reported that linolenic acid may account for up to 96% of the fatty acid content of galactolipids for some species (Douce and Joyard, 1980). In general, linolenic acid accounts for 87% of the fatty acyl residues of DGDG and 72% of the fatty acyl residues of MGDG (Douce and Joyard, 1980), but accounts for less than 20% of the fatty acid content of most other membranes in plant cells (Harwood, 1980). SQDG and PG of chloroplasts, contain a larger proportion of saturated fatty acids than MGDG and DGDG.

#### *1.2.3.2 Thylakoid Proteins*

Thylakoids differ from other cellular membranes in that they have a higher protein-to-lipid ratio (de Boer and Weisbeek, 1991). There are four major protein complexes localized in the thylakoid: photosystems I and II, the cytochrome $b_6/f$ complex and the ATP synthase complex. All are multi-protein complexes and contain polypeptides encoded by both the nuclear and plastid genomes (Wollman et al., 1999). The coordinated expression of thylakoid protein genes by the two genomes is required for efficient assembly of functional photosynthetic membranes (Schnell et al., 1998). The majority of thylakoid proteins (and chloroplast proteins as a whole) are encoded by nuclear genes and synthesized in the cytosol. These proteins are synthesized as higher molecular weight precursors and targeted for import into the chloroplast by cleavable N-terminal transit sequences (Schnell, 1998; Keegstra and Cline, 1999). Once inside the chloroplast, proteins destined for the thylakoid can use one of four pathways of import into the photosynthetic membrane (Robinson et al., 1998). All of the identified mechanisms require the assistance of other chloroplast proteins for targeting and insertion, and two of the four are energy-dependent processes (Robinson et al., 1998). Integration of proteins

into thylakoids is made even more complex when one considers that proteins encoded by the plastid genome may use yet another mechanism for insertion. Specifically, many plastid-encoded proteins are co-translationally inserted into thylakoids, rather than post-translationally inserted as are the nuclear-encoded proteins (Minami and Watanabe, 1984).

The most abundant thylakoid proteins are the chlorophyll *a-b* binding proteins of the light-harvesting complex (LHC) (Hoober et al., 1994). Nuclear *cab* genes (recently renamed *lhcb* genes) encode the LHC proteins, which are bound to chlorophyll molecules *in situ*. The complex contains the majority of thylakoid chlorophyll, and funnels absorbed light energy to the photosystem reaction centers to drive photosynthesis. The complex is often thought of as the fifth major protein complex of the thylakoids.

#### *1.2.3.3 Chlorophyll*

Perhaps the most characteristic component of thylakoid membranes (and chloroplasts) is chlorophyll, the pigment that makes leaves appear green. All chlorophyll molecules are found in thylakoids as complexes with proteins. Most chlorophyll is located in the light-harvesting complexes coupled with LHC proteins, while some of the pigment molecules are also components of the reaction centers of PSII and PSI (Melis, 1991). Recently, it has been demonstrated that chlorophyll is also a minor component of the cytochrome $b_6/f$ complex (Huang et al., 1994). The primary function of chlorophyll is to capture light energy, which is then used to drive photophosphorylation.

## 1.3 RUBISCO

Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco, E.C. 4.1.1.39) is the major soluble leaf protein in plants (Miziorko and Lorimer, 1983). In fact, the enzyme comprises approximately 50% of the total soluble protein in the chloroplasts of $C_3$ leaves, and is thought to be one of the most abundant proteins on earth (Gepstein, 1988). While most Rubisco is found in the stroma, a small amount has been found loosely associated with thylakoids both independently (Makino and Osmond, 1991; Mori et al., 1984), and as part of multienzyme Calvin cycle complexes containing Rubisco (Suss et al., 1993; Suss et al., 1995). It not only catalyzes the first step in the carbon fixation cycle (Calvin cycle), but also acts as an oxygenase in the primary step of photorespiration (Miziorko and Lorimer, 1983). It is these two competing reactions that determine the net photosynthetic yield of many higher plants (Roy, 1989).

Rubisco catalyzes the carboxylation of the five-carbon compound D-ribulose-1,5-bisphosphate (RuBP), yielding two molecules of the three-carbon compound D-3-phosphogylcerate (3-PGA) (Miziorko and Lorimer, 1983). The reaction thus results in the net gain of carbon, and the 3-PGA molecules are converted into usable sugars via the other reactions of photosynthesis. The enzyme also catalyzes the oxygenation of RuBP, which is the primary event in the photorespiratory carbon oxidation cycle (Miziorko and Lorimer, 1983). Carbon dioxide not only acts as a substrate for carboxylation and a competitive inhibitor of oxygenation, but also as an activator of the enzyme for both carboxylation and oxygenation (Miziorko and Lorimer, 1983). The condensation of $CO_2$ forms a carbamate with the enzyme, which is stabilized by the catalytically essential $Mg^{2+}$ (Hartman and Harpel, 1994). Therefore, both $CO_2$ and $Mg^{2+}$ must activate Rubisco for both of its catalytic functions.

Rubisco activity is regulated by an enzyme termed Rubisco activase and an inhibitor, 2'-carboxy arabinitol 1-phosphate (2CA1P). 2CA1P accumulates in the dark and binds to the active site of the enzyme, and is presumed to stabilize the activated enzyme as photosynthesis slows (Gutteridge and Gatenby, 1995). The process of 2CA1P release from the active site and activation of the enzyme is assisted by Rubisco activase. RuBP, the natural substrate, can also prevent activation of Rubisco from its inactivated state (i.e. when the active site is free of co-factors and substrate) by preventing access of activating cofactors to their binding sites (Gutteridge and Gatenby, 1995).

Rubisco holoenzyme is a hexadecameric polypeptide of about 560 kDa, consisting of eight large and eight small subunits ($L_8S_8$) in plants and blue-green algae (Andrews and Lorimer, 1987). However, in photosynthetic bacteria such as *Rhodospirillum rubrum*, Rubisco is found as a homodimer of two 50-kDa large subunits ($L_2$) (Hartman and Harpel, 1994).

### 1.3.1 Rubisco Large Subunit (RLSU)

The *rbcL* gene encodes the large subunit of Rubisco (RLSU), and is present as a single copy per chloroplast genome. RLSU is synthesized on 70S chloroplast ribosomes (Berry et al., 1985) and has a molecular weight in the range of 52-56 kDa (Langridge, 1981). Since there are up to 100 copies of cpDNA per chloroplast and a single leaf cell may contain 200 or more chloroplasts, it follows that there may be several thousand copies of the large subunit gene per leaf (Miziorko and Lorimer, 1983). Langridge (1981) reports that the large subunit of spinach is synthesized as a precursor polypeptide approximately 1-2 kDa larger than mature large subunit found in the holoenzyme. Indeed, an amino-terminal fragment of about 14 amino acids is post-translationally removed from RLSU, yielding the mature polypeptide that is

incorporated into $L_8S_8$ Rubisco (Amiri et al., 1984). Many precursor proteins that differ only slightly from their mature molecular weight are inserted into, or transported across, a membrane (Langridge, 1981). Insertion into membranes occurs when a polypeptide bears an appropriate signal sequence of amino acids at its amino-terminal end, which is cleaved off upon insertion to yield the mature protein (Singer, 1990). Hattori and Margulies (1986) report that a portion of large subunit translation within chloroplasts occurs on polyribosomes associated with thylakoids. It is known that such thylakoid-bound ribosomes are involved in the co-translational insertion of polypeptides that are localized in the thylakoids (Hattori and Margulies, 1986). The well-established fact that RLSU is a hydrophobic polypeptide (Gatenby, 1984) supports the idea that it may also be co-translationally inserted into thylakoids by membrane-bound polysomes. Hattori and Margulies (1986) also suggest that since the formation of $L_8S_8$ Rubisco is a multi-step process yet to be fully elucidated, a membrane-associated step could potentially be involved.

### 1.3.2 Rubisco Small Subunit (RSSU)

The nuclear-encoded small subunit of Rubisco (RSSU) is synthesized on free, cytoplasmic ribosomes as a 20 kDa precursor protein (Berry et al., 1985). A transit sequence of 57 amino acids is cleaved from the amino-terminus of the precursor during transport into chloroplasts, yielding the mature, 12-15 kDa polypeptide in the stroma (Chua and Schmidt, 1978; Langridge, 1981; Hoober, 1987). The exact mechanism of small subunit action in the holoenzyme remains unclear. It is known that the large subunits contain the catalytically active site of the holoenzyme. However, it is unclear if the large subunits possess activity without small subunits also bound to them (Andrews and Lorimer, 1987).

A family of two to 12 *rbcS* genes in the nuclear genome (Berry et al., 1985; Gutteridge and Gatenby, 1995) codes for RSSU. The number of genes involved varies among species. While at least one of these genes is always expressed, two or more are often expressed simultaneously (Hoober, 1987), and different genes may be expressed at different levels at the same time. In addition, expression is strongly induced by light (Gutteridge and Gatenby, 1995).

### 1.3.3 Rubisco Holoenzyme Assembly

The assembly of large and small subunits into Rubisco holoenzyme ($L_8S_8$) occurs in the stroma after the import and processing of RSSU (Gatenby, 1984). It is a complex process that requires the coordinated expression of two genomes and the assistance of proteins called chaperonins (Roy, 1989). Chaperonins comprise a family of proteins that fall into the larger class of proteins termed molecular chaperones. Molecular chaperones include proteins such as heat-shock protein 70 (hsp70), which is active in times of stress (e.g. heat shock), although not all molecular chaperones are heat-shock proteins (Hartman and Harpel, 1994). Chaperonins regulate protein folding by stabilizing folding intermediates, thereby influencing the kinetic partitioning between aggregated (misfolded) and correctly folded proteins (Gutteridge and Gatenby, 1995). In this way, they promote assembly of subunits into functional complexes without themselves forming part of the final structure (Roy, 1989). Most large subunits are found in association with "Rubisco-binding protein", now called plastid chaperonin 60 (cpn60) (Hartman and Harpel, 1994). This major stromal protein is functionally analogous to the *E. coli* protein, groEL, but differs in that it is composed of equal quantities of two slightly different polypeptides, cpn60-$\alpha$ and cpn60-$\beta$ (Martel et al., 1990). Plastid cpn60 also binds

newly imported RSSU (Hartman and Harpel, 1994; Gutteridge and Gatenby, 1995). A chloroplast co-chaperonin that is analogous to groES of *E. coli*, is also involved in the assembly of $L_8S_8$ Rubisco (Viitanen et al., 1995). Interestingly, chloroplast co-chaperonin in fact consists of 21-kDa "double domains" rather than single 10-kDa domains as in *E. coli* (Baneyx et al., 1995; Viitanen et al., 1995), and is therefore referred to as chloroplast cpn21 (Gutteridge and Gatenby, 1995).

The details of assembly are still not fully elucidated. However, current models propose that KCl and MgATP are required along with the chaperonins. It is also believed that assembly proceeds via the formation of large subunit dimers ($L_2$) and an eight-large-subunit ($L_8$) core before addition of the eight small subunits (Hartman and Harpel, 1994; Roy and Gilson, 1997). The major obstacle to elucidating the assembly pathway of this essential photosynthetic enzyme is the failure to reconstruct the pathway *in vitro* (Gutteridge and Gatenby, 1995). Specifically, the difficulty resides in successful formation of the soluble $L_8$ core of the enzyme from the individual, insoluble large subunits (Gutteridge and Gatenby, 1995).

## 1.4 CYTOCHROME *f*

Cytochrome *f* is a major intrinsic thylakoid membrane protein and a component of the cytochrome $b_6/f$ complex. The other subunits of the complex include cytochrome $b_6$ (encoded by the plastid *petB* gene), the Rieske-FeS protein (encoded by the nuclear *petC* gene), subunit IV (encoded by the plastid *petD* gene) and three smaller subunits of less than 4 kDa each, namely the PetM, PetG and PetL polypeptides (Cramer et al., 1996; Hager et al., 1999). An eighth subunit composed of only 29 amino acids has recently been discovered, which is

encoded by the *petN* gene of the plastid genome (Hager et al., 1999). Interestingly, the cytochrome $b_6/f$ complex has been found to contain one tightly bound chlorophyll *a* molecule, and more recently it has been demonstrated that β-carotene is also part of the complex (Cramer et al., 1996; Zhang et al., 1999). Cytochrome $b_6/f$ is found in the thylakoid as a functional dimer, and is evenly distributed between the grana and stroma lamellae of the membrane (Albertsson, 1995; Cramer et al., 1996). The complex is part of the photosynthetic electron transport chain, transferring electrons from PSII to PSI in linear electron flow. Cytochrome *f* accepts single electrons indirectly from PSII through the lipid-mobile molecule, plastoquinone and another member of the cytochrome $b_6/f$ complex, the Rieske FeS subunit, and transfers them to plastocyanin (Gray, 1992). Plastocyanin is a soluble, mobile protein of the lumen that transfers electrons to PSI. Accompanying electron transfer through the cytochrome $b_6/f$ complex, is proton translocation from the stroma into the thylakoid lumen (Hope, 1993; Huang et al., 1994; Chain and Malkin, 1995). Therefore, the cytochrome $b_6/f$ complex plays an important role in establishing the proton motive force that is used to drive synthesis of ATP by the ATP synthase (Hope, 1993). The cytochrome $b_6/f$ complex is also involved in cyclic electron transfer in conjunction with ferredoxin, plastoquinone, and PSI (O'Keefe, 1988; Hope, 1993).

The *petA* gene of the chloroplast genome encodes cytochrome *f*. The protein is synthesized on thylakoid-bound polysomes and co-translationally inserted into the thylakoid membrane via the SecA-dependent pathway (Gray et al., 1984; Nohara et al., 1996; Mould et al., 1997). Indeed, cytochrome *f* is synthesized as an apoprotein with a 35 amino acid cleavable, thylakoid-targeting signal peptide on its N-terminus. The mature protein is 285 amino acids in length and is highly conserved among higher plant species, with over 80% of

the residues invariant (Gray, 1992). The protein was first discovered in 1938, but not reported in detail until 1951 by Hill and Scarisbrick (Gray, 1992). It was named cytochrome *f*, after the Latin (*frons*) or French (*feuille*) for leaf, and because cytochromes *a* to *e* had already been described (cytochrome *e* was subsequently determined to be identical to the previously identified cytochrome $c_1$, and the name is no longer used) (Gray, 1992; Cramer et al., 1994; Prince and George, 1995).

The protein is anchored in the thylakoid membrane by a single 20 amino acid trans-membrane α-helix near the C-terminal end (residues 251-270) (Willey et al., 1984). The extreme 15 C-terminal residues protrude into the stroma, while the 250 residues N-terminal to the α-helix comprise the large hydrophilic, globular domain of the protein that extends into the thylakoid lumen. This large globular domain houses the haem that is ligated to cytochrome *f* (Gray, 1992). Residues $Cys_{21}$-$His_{25}$ act as the haem-binding site.

## 1.5 LIPID BODIES

Lipid bodies are important for energy storage and/or lipid transport in most eukaryotes and are formed at some point in the life cycle of mammals, insects, plants and microorganisms (Spector et al., 1981; Murphy and Vance, 1999). Storage and transport lipids are most often neutral lipids such as triacylglycerol and are contained within spherical droplets (lipid bodies) that range in diameter from 0.1 to 50 μm. The neutral lipid component comprises a hydrophobic core of lipid bodies, which is circumscribed by a monolayer of polar lipids, oriented such that the hydrophilic head groups come in contact with the aqueous surroundings. Lipid bodies from many different cell types have been described. Only those relevant to the current research will be discussed here.

### 1.5.1 Oil Bodies from Seeds and other Organs

Seeds of many plant species store triacylglycerols (TAGs) as energy reserves to be used during germination and postgerminative growth (Huang, 1996). Indeed, lipids provide the most energy on a per weight basis of any macromolecule. TAGs are stored in small oil bodies of approximately 0.5-2.0 μm in diameter. Seed oil bodies are perhaps the best-characterized types of plant lipid body. They are surrounded by a monolayer of phospholipids, whose hydrophobic acyl chains project into the interior of the particle where they interact with the triacylglycerol core. Oil bodies are stable even upon isolation, due in part to the presence of the protein oleosin. Oleosins are small proteins of 15 to 26 kDa that possess the longest consecutive stretch of hydrophobic amino acid residues (72) of any known protein (Huang, 1996). This hydrophobic domain is highly conserved among oleosins from diverse species and occupies the central portion of the protein (Murphy, 1993). It penetrates through the phospholipid monolayer into the hydrophobic TAG matrix, anchoring the oleosin onto the oil body. The amphiphilic carboxy and amino termini of the protein are not well conserved, and associate with the polar head groups of the phospholipids to coat the entire surface of the oil body.

Oil bodies are formed during the final stages of seed development, and arise from specific microdomains of the endoplasmic reticulum that contain TAG biosynthetic enzymes (Murphy and Vance, 1999). Oleosin is thought to be co-translationally inserted into the ER, but is not necessarily involved in the formation of the oil bodies as not all species that produce oil-bearing seeds contain oleosin. Rather, oleosin is thought to play a role in preventing coalescence of oil bodies during extreme water stress such as dehydration and sudden

rehydration of almost completely dry seeds (Murphy and Vance, 1999). In addition, species that do not produce oleosin possess much larger oil bodies than those species that do produce the protein (termed dessication-tolerant species), suggesting that oleosin regulates the size of lipid bodies.

Oleosin is not only produced in seeds. Tapetal cells are another major lipid-producing cell type that also contain oleosin (Ting et al., 1998; Hernández-Pinzón et al., 1999). Specifically, oleosins are localized on the TAG-containing lipid bodies of the tapetal cells. Tapetal cells form the single cell layer that nourishes developing microspores, and they also provide the final protein and lipid coat for pollen grains (Murphy and Vance, 1999; Hernández-Pinzón et al., 1999).

### 1.5.2 Lipid-Protein Particles

Lipid-protein particles localized in the cytosol were first described for cotyledons of etiolated *Phaseolus vulgaris* seedlings, and were initially referred to as deteriosomes (Yao et al., 1991a). They have since been isolated from a number of different tissues, including senescing carnation petals, rat liver cytosol and insect intestine (Yao et al., 1991b; Yao et al., 1993; Desantis et al., 1995; McKegney et al., 1995; Hudak and Thompson, 1996). Lipid-protein particles can be isolated by ultrafiltration of the supernatant following high-speed centrifugation of cytosol or by flotation centrifugation (Thompson et al., 1997). Isolated particles resemble those visualized *in situ*, in that they range from 30 to 300 nm in diameter and are uniformly osmiophilic in thin section (Thompson et al., 1998). They are enriched in membrane proteins and their metabolites as well as membrane lipids and their metabolites, prompting the suggestion that they are involved in the removal of these metabolites from their

membranes of origin. Indeed, if not removed from the membrane, metabolites of proteins and lipids could compromise the structure of the bilayer.

It has been suggested that these destabilizing metabolites phase-separate within the plane of the bilayer into distinct domains (Thompson et al., 1997). These domains of concentrated lipid and protein metabolites are then thought to be removed from the membrane through a process of blebbing or budding, in association with membrane lipids and possibly with the assistance of unidentified proteins, in a manner reminiscent of that proposed by Murphy and Vance (1999) for oil body release from the endoplasmic reticulum. The resulting lipid particles are thought to be surrounded by a monolayer of polar lipid and contain a core of neutral lipid and lipid metabolites (Thompson et al., 1998). In keeping with the proposed role of lipid-protein particles in removing destabilizing lipid and protein metabolites from membranes, it is noteworthy that in non-senescing tissue, metabolites formed during normal membrane turnover do not accumulate in membrane bilayers. Rather, they are present in cytosolic lipid-protein particles (Thompson et al., 1998). This has prompted the proposal that formation of lipid-protein particles is an integral feature of membrane turnover (Thompson et al., 1998).

With advancing senescence, the formation of cytosolic lipid-protein particles becomes impaired, which leads to an accumulation of lipid metabolites in membranes and a consequent disruption of bilayer structure (Thompson et al., 1998). Indeed, lipid-protein particles are less abundant in senescing tissue than in young tissue (Yao et al., 1991b; Hudak et al., 1995), and phase-separating lipid metabolites have been shown to accumulate in senescing membranes (Yao et al., 1991b; Thompson et al., 1998).

Lipid-protein particles analogous to those present in the cytosol have also been isolated from bean leaf chloroplasts. Like their cytosolic counterparts the chloroplastic particles are spherical, 150-300 nm in diameter, osmiophilic in thin section and enriched in membrane metabolites, particularly free fatty acids (Ghosh et al., 1994). However, these particles differ from those isolated from cytosol in that they contain metabolites of photosynthetic proteins and galactolipids rather than phospholipids (Ghosh et al., 1994). The chloroplastic particles can be isolated from stroma, and are thought to originate from thylakoids and play an integral role in thylakoid turnover by voiding destabilizing metabolites from the membrane (Ghosh et al., 1994). The similarity between stromal lipid-protein particles and plastoglobuli has been recognized, but so far not investigated (Ghosh et al., 1994; Thompson et al., 1998). However, differences in size and apparent differences in function have prompted speculation that the two particle types are generically related but functionally distinct (Thompson et al., 1998). Specifically, lipid-protein particles are thought to play a role in thylakoid turnover, whereas plastoglobuli appear to be involved in thylakoid dissolution during senescence.

### 1.5.3 Plastoglobuli

Plastoglobuli are lipid bodies found in all plastid types, including chloroplasts. First reported in 1961 (Park and Pon, 1961), plastoglobuli from many species including *Phaseolus vulgaris* have been described, yet their exact structure and composition remain elusive (Barton, 1966; Lichtenthaler, 1968; Hansmann and Sitte, 1982; Steinmüller and Tevini, 1985a; Pozueta-Romero et al., 1997). It is generally accepted that plastoglobuli consist mainly of plastoquinone, α-tocopherol and triacylglycerol (TAG) (Bailey and Whyborn, 1963; Lichtenthaler, 1969; Steinmüller and Tevini, 1985a). Plastoglobuli of chloroplasts do not

contain chlorophyll, although traces of carotenoids have been detected (Lichtenthaler, 1969). In contrast, the plastoglobuli of chromoplasts are enriched in carotenoids (Hansmann and Sitte, 1982; Steinmüller and Tevini, 1985a). Interestingly, it has been recently demonstrated that the major neutral lipid of plastoglobuli from tapetal cell elaioplasts is sterol esters (Hernández-Pinzón et al., 1999). As chloroplasts age, the composition of plastoglobuli becomes slightly altered. Specifically, carotenoids, free fatty acids and phytol, which is apparently released from chlorophyll by the action of chlorophyllase, become more prevalent constituents (Steinmüller and Tevini, 1985b; Matile et al., 1999). While there is general consensus that plastoglobuli contain neutral lipid, it is not universally accepted that plastoglobuli are circumscribed by a polar lipid monolayer in the same way that a phospholipid monolayer surrounds oil bodies (Murphy, 1993; Huang, 1996). This uncertainty stems from conflicting reports about whether the plastoglobuli contain galactolipid and the difficulty of visualizing a half-unit membrane by electron microscopy (Greenwood et al., 1963; Simpson and Lee, 1976; Hansmann and Sitte, 1982; Steinmüller and Tevini, 1985a). However, recent evidence obtained from the study of tapetal cell elaioplasts suggests that it is probable that a monolayer of galactolipids surrounds the neutral lipid core of plastoglobuli and that these lipid bodies originate from thylakoids (Hernández-Pinzón et al., 1999). This evidence is in agreement with a model for plastoglobuli organization proposed by Knoth et al. (1986).

There have also been conflicting reports regarding the protein content of plastoglobuli. It has been argued that proteins associated with isolated plastoglobuli are artifactual (Steinmüller and Tevini, 1985a), but others maintain that numerous proteins are authentic constituents of these lipid bodies (Bailey and Whyborn, 1963; Hansmann and Sitte, 1982; Kessler et al., 1999; Hernández-Pinzón et al., 1999). Some of these proteins have been recently characterized and

found to be members of a family of lipid-binding proteins (Chen et al., 1998). Included in this family are plastid-lipid associated protein (PAP or PLP), fibrillin, plastoglobulin 1 (PG1), carotenoid-associated protein (CHRC), carotene globule protein (Cgp), and the 32- and 34-kDa chloroplast drought-induced stress proteins (CDSP 32 and CDSP 34) (Deruère et al., 1994; Katz et al., 1995; Vishnevetsky et al., 1996; Pozueta-Romero et al., 1997; Kessler et al., 1999; Eymery and Rey, 1999; Hernández-Pinzón et al., 1999). All of these proteins are specifically associated with plastid lipid, including plastoglobuli of chloroplasts, chromoplasts and elaioplasts, as well as thylakoids and fibrils. They range in size from 30-38 kD, and it has been suggested that they may be involved in maintaining the structural stability of plastid lipid bodies in much the same way that oleosin functions in oil bodies (Pozueta-Romero et al., 1997; Chen et al., 1998; Ting et al., 1998). This suggestion stems, in part, from the finding that members of the PAP/fibrillin family are localized to the periphery of plastoglobuli (Pozueta-Romero et al., 1997; Kessler et al., 1999). In light of accumulating evidence for their existence, it seems that these proteins are not only genuine components of plastoglobuli, but can be considered specific markers for these plastid lipid bodies.

The exact role of plastoglobuli has not yet been conclusively determined (Lichtenthaler, 1968; Tuquet and Newman, 1980). However, it is assumed, based on their reduction in size and number during the biogenesis of thylakoids and their subsequent accumulation and increase in size during thylakoid degradation, that they act as storage pools for thylakoid components, especially those liberated during breakdown of the membrane (Sprey and Lichtenthaler, 1966; Lichtenthaler, 1968; Lichtenthaler and Weinert, 1970). Indeed, plastoglobuli isolated from senescing beech leaves were found to be enriched in free fatty acids, lipid catabolites released from the membrane by the action of lipases (Steinmüller and

Tevini, 1985b). It has also been suggested that plastoglobuli may function as sites of surplus lipid storage (Greenwood et al., 1963; Thomson and Platt, 1973). More recently, it has been reported that plastoglobuli of senescing soybean chloroplasts are exuded through the envelope into the cytoplasm, where they are degraded (Guiamét et al., 1999).

Based on differences in lipid composition between plastoglobuli of chloroplasts and those of chromoplasts and even among chloroplastic plastoglobuli, it has been suggested that there are distinguishable subpopulations of plastoglobuli (Simpson and Lee, 1976). In an earlier study, Bailey and Whyborn (1963) presented evidence that two classes of plastoglobuli exist in the chloroplasts of *Beta vulgaris* leaves, which differ primarily in their lipid content and can be separated based on differences in density. Plastoglobuli of differing densities have also been isolated from the chloroplasts of *Pisum sativum* leaves using a sucrose gradient (Kessler et al., 1999). In addition, the presence of plastoglobuli with different electron densities within chloroplasts of *Capsicum annuum* leaves is thought to reflect compositional and perhaps functional differences among the particles (Simpson and Lee, 1976).

## 1.6 TURNOVER AND SENESCENCE IN CHLOROPLASTS

The majority of leaf lipid and protein is localized within chloroplasts, and hence these organelles are an important source of nutrients that can be recycled to growing parts of the plant during leaf senescence. In addition, chloroplast components are regularly replaced as they become damaged by newly synthesized components, a process known as turnover, which helps to ensure that the functions of the organelle are maintained (Woolhouse, 1987; Matile, 1992). Indeed, all cellular components are regularly turned over as part of normal cell function. The process of turnover is particularly important in the maintenance of thylakoid

function, as the constant input of light energy and high oxygen concentration resulting from photosynthesis combine to generate high levels of reactive oxygen species, molecules that are particularly damaging to membranes. Surprisingly, however, relatively little is known about the processes that facilitate the degradation of chloroplast components or how degradation is regulated. Indeed, Matile (1992) states that "nearly nothing is known about the catabolic aspects of turnover" in chloroplasts.

The earliest and most obvious structural changes associated with leaf senescence occur within chloroplasts (Woolhouse, 1984; Gepstein, 1988). Senescing chloroplasts have been termed gerontoplasts, reflecting the fact that their metabolism is strictly catabolic (Matile, 1992). Thylakoids undergo dramatic change during chloroplast senescence. The membranes become dilated and disorganized at the onset of senescence and, together with stromal components, are extensively degraded as gerontoplast development proceeds. This degradation causes the volume of senescing chloroplasts to shrink. Coincident with thylakoid degradation, the size and abundance of plastoglobuli increase. The plastid envelope persists until the very late stages of senescence. Thus, a fully developed gerontoplast is somewhat smaller than a chloroplast and consists of an envelope surrounding a number of large plastoglobuli (Lichtenthaler, 1968; Matile et al., 1999). The maintenance of envelope integrity until the latter stages of senescence is essential for the orderly export of chloroplast components destined to be recycled to other parts of the plant (Woolhouse, 1984). Recycling of nutrients is perhaps the most important feature of chloroplast senescence.

Hormones play an integral role in regulating leaf and chloroplast senescence. Specifically, hormonal signals likely lead to the up-regulation of senescence-associated genes and the down-regulation of other genes via activator proteins that are themselves activated either directly or

indirectly by the hormones. Among the five classic phytohormones, abscisic acid, cytokinins and ethylene have the most profound effects. In fact, many factors cooperate to influence the timing of senescence.

The gaseous phytohormone, ethylene, plays a particularly prominent role in the regulation of senescence in dicotyledonous species (Smart, 1994). In particular, it accelerates many of the physiological changes associated with senescence including chlorophyll loss (Mattoo and Aharoni, 1988). Studies on antisense and mutant plants showing delayed senescence have indicated that ethylene alters the timing of senescence (Buchanan-Wollaston, 1997), and it has been proposed that ethylene accelerates senescence by activating senescence-associated genes while repressing the expression of photosynthesis-associated genes (Grbic and Bleeker, 1993), but only in leaves that are already programmed to begin senescing. Abscisic acid (ABA) has also been found to accelerate leaf senescence in a wide range of species, particularly when applied exogenously to detached leaves. Endogenous ABA levels also increase at the onset of senescence in detached leaves, and under conditions of stress such as drought. The jasmonates, jasmonic acid and methyl jasmonate, are derived from fatty acids and have been shown to promote senescence. The exact role of jasmonates in senescence is not clear. However, it has been suggested that they are part of a stress/senescence signal transduction pathway leading to the expression of senescence-associated genes, rather than being a direct cause of senescence (Smart, 1994).

Cytokinins play an essential role in the regulation of leaf senescence, in that they dramatically delay the process in some species (Gan and Amasino, 1995). Application of exogenous cytokinins to detached leaves can cause regreening in some species and delays senescence in others, and endogenous cytokinin levels have been shown to decrease in leaves

at the onset of senescence in both attached and detached leaves. It has, therefore, been proposed that senescence can only be initiated when cytokinin levels fall below a threshold level (Buchanan-Wollaston, 1997).

### 1.6.1 Chlorophyll Turnover and Degradation during Senescence

The net loss of chlorophyll from thylakoid membranes is the most easily recognized symptom of leaf senescence, and signals the transition of chloroplasts to gerontoplasts (Matile et al., 1999). The degradation of chlorophyll unmasks the carotenoids that persist in thylakoids, leading to the yellowing of leaves, and is considered a reliable measure of leaf senescence (Gepstein, 1988). It is believed that the first step in the degradation of chlorophyll during senescence is catalyzed by chlorophyllase, which hydrolyzes chlorophyll into chlorophyllide and free phytol. Other enzymes of the pathway include Mg-dechelatase, pheophorbide *a* oxygenase and RCC (Red Chlorophyll Catabolite) reductase. Together, these enzymes convert chlorophyllide into linear tetrapyrollic structures termed nonfluorescent chlorophyll catabolites (Matile et al., 1999). While the biochemistry of chlorophyll catabolism in senescing leaves is fairly well established, only recently have the first chlorophyllase genes been isolated (Matile et al., 1999; Tsuchiya et al., 1999).

Little is known about the rates and regulation of chlorophyll turnover in non-senescing leaves (Thomas, 1997; Matile et al., 1999). Although the pathway of chlorophyll biosynthesis is well established (Thomas, 1997), the mechanism for the breakdown of chlorophyll during the catabolic phase of the turnover cycle is not well understood. However, it is believed that the system responsible for chlorophyll catabolism during turnover is different from that operating during senescence (Matile et al., 1999).

### 1.6.2 Protein Turnover and Degradation during Senescence

While not as readily apparent to the casual observer, the degradation of chloroplastic protein during leaf senescence is a more significant and important process than is chlorophyll degradation (Matile, 1992). Indeed, protein represents a much larger portion of the recyclable material contained in chloroplasts than does chlorophyll. Specifically, chloroplastic proteins are an excellent source of nitrogen that can be reclaimed and used by the growing parts of the plant. Although Rubisco is the major source of this nitrogen, there are significant levels of proteins associated with thylakoids that contribute to the recycling of nitrogen as well. For example, a selective loss of cytochrome $b_6/f$ complex polypeptides from thylakoids has been reported during senescence (Ben-David et al., 1983; Roberts et al., 1987). The selective loss of cytochrome $b_6/f$ is mainly responsible for the decline in linear electron transport observed during senescence (Jenkins and Woolhouse, 1981; McRae et al., 1985). Levels of other thylakoid proteins such as the abundant light-harvesting chlorophyll *a*/*b*-binding protein decline significantly during leaf senescence as well (Bate et al., 1991; Matile, 1992). In addition, the 32 kDa D1 protein of photosystem II is rapidly turned over at all stages of chloroplast development, and this continues throughout senescence (Mattoo et al., 1984; Roberts et al., 1987; Bate et al., 1991). Indeed, the entire protein population of plants is in a dynamic state of flux (Peoples and Dalling, 1988).

D1 turnover entails a number of steps. Initially, light-harvesting complexes associated with PSII reaction centers must dissociate, which allows for migration of the PSII complex to the stromal lamellae (Melis, 1991). The damaged D1 protein, which appears to be phosphorylated, is exposed to a membrane-bound protease and can be removed (Melis, 1991;

Aro et al., 1993; Buetow, 1997). The enzymes involved in D1 protein turnover have not been identified (Buetow, 1997), although it is known that a 23.5 kDa catabolite is formed by a protease thought to be associated with thylakoids early in the process of D1 turnover (Greenberg et al., 1987, Mattoo et al., 1989). After removal of the damaged protein, a new D1 polypeptide is co-translationally inserted into the thylakoid and incorporated into the PSII complex. Following light-dependent activation of the repaired PSII, the complex associates with a LHC and migrates back to the granal region of the thylakoid (Aro et al., 1993). It has been proposed that elucidating the complete mechanism of D1 turnover may have general implications for understanding chloroplast proteolysis (Woolhouse, 1984; Gepstein, 1988).

A number of chloroplastic proteases have been described (Dalling and Nettleton, 1986; Buetow, 1997). However, the specific roles for many of these proteases remain obscure, as most have only recently been identified (Gepstein, 1988). For example, it is not clear if the enzymes involved in normal turnover of proteins such as D1 are the same as those involved in protein degradation during thylakoid senescence. As well, it has been suggested that a cytochrome *f*-specific protease is associated with thylakoids, although it has not yet been isolated (Gray et al., 1994). Although many reports on the enzymes responsible for the degradation of chloroplast components suggest that they are up-regulated in compartments spatially separate from the chloroplast (Guimét et al., 1999), a recent report identifies a Clp protease that is up-regulated in chloroplasts during leaf senescence (Nakabayashi et al., 1999).

Finally, it has not yet been conclusively determined how damaged membrane proteins are removed from thylakoids. However, it has been suggested that it may occur in association with lipids (Thomas and Hilditch, 1987), and the more recent finding that lipid-protein particles isolated from the stroma are enriched in many thylakoid proteins and their catabolites

supports this contention (Ghosh et al., 1994). Indeed, it has been suggested that these lipid-protein particles serve as a vehicle for removing misfolded proteins and their catabolites from the membrane that would destabilize the bilayer if allowed to accumulate (Ghosh et al., 1994; Thompson et al., 1998). In addition, a recent report describes plastoglobuli from soybean leaves that contain CP47 and LHC polypeptides from thylakoids (Guimét et al., 1999).

### 1.6.3 Lipid Turnover and Degradation during Senescence

Degradation of thylakoids involves extensive loss of phospholipids and galactolipids. Phospholipids are degraded by the concerted actions of phospholipase D, phosphatidic acid phosphatase, lipolytic acyl hydrolase and lipoxygenase (Thompson et al., 1998). Phosphatidic acid is one of the products of phospholipase D action on phospholipids, and is converted to diacylglycerol (DAG) by phosphatidic acid phosphatase. DAG is a membrane-destabilizing molecule (Allan et al., 1976) that is further degraded by lipolytic acyl hydrolase to produce free fatty acids (Thompson et al., 1998). Polyunsaturated fatty acids such as linolenic acid, which is particularly abundant in thylakoids, then serve as substrates for lipoxygenase, which can initiate lipid peroxidation and the formation of reactive oxygen species (Holden, 1970; Thompson, 1988). The fate of degraded lipids in chloroplasts is not entirely clear, but it is believed that some of the acyl moieties released during lipid breakdown are respired and used as energy for fuelling the senescence process (Matile, 1992). As well, it appears that much of the lipid from senescing thylakoids is transferred to and stored in plastoglobuli, since the size and number of these lipid bodies increase in parallel with thylakoid dissolution (Sprey and Lichtenthaler, 1966). Significant loss and deacylation of galactolipids has been observed during chloroplast degradation (Fong and Heath, 1977; Woolhouse, 1984). In fact, the

galactolipid content of leaves declines more rapidly than the phospholipid content (Tevini, 1976; Fong and Heath, 1977). Surprisingly, though, the enzymes responsible for galactolipid degradation have not been extensively studied (El-Hafid et al., 1989).

Although the exact mechanism of thylakoid breakdown has yet to be elucidated, it does seem that senescence of these membranes differs from that of other cellular membranes (Thompson, 1988). Specifically, senescence of thylakoids is not accompanied by the extensive loss of lipid fluidity that characterizes senescence of other membranes (McRae et al., 1985). The reason for this difference is not entirely clear, but is presumably linked to the requirement of photosynthesis for a highly fluid membrane (Douce and Joyard, 1980; Quinn, 1997). Indeed, photosynthesis continues until the late stages of chloroplast senescence, albeit at a reduced rate (Matile, 1992). Premature loss of thylakoid fluidity would likely lead to a more rapid decline in photosynthetic capacity. The mechanism responsible for maintaining thylakoid fluidity in senescing thylakoids has not been investigated. However, it is possible that the formation of plastoglobuli during senescence and the consequent removal from thylakoids of lipid metabolites such as free fatty acids that may contribute to the decrease in lipid fluidity, may be involved.

Thylakoid lipids are turned over once they become damaged, or when there is a need to alter the lipid composition of the membrane due to stress such as drought (Long et al., 1994). The synthesis of thylakoid lipids has been largely elucidated (Douce and Joyard, 1990). Fatty acids are synthesized within the chloroplast (Douce and Joyard, 1990), while thylakoid galactolipids (MGDG and DGDG) are synthesized on envelope membranes and transferred to thylakoids in vesicles (Hoober et al., 1994). The catabolic aspect of lipid turnover is not well studied. However, it presumably involves the action of lipid-degrading enzymes similar to

those involved in thylakoid lipid degradation during senescence. It remains to be determined whether the enzymes involved are the same, or if a different subset of isoforms is responsible for lipid degradation during turnover.

#### *1.6.3.1 Role of Lipases*

The principle enzyme involved in thylakoid lipid breakdown is galactolipase (E.C. 3.1.1.26). Galactolipases have been purified from a number of leaf homogenates, and have been shown to be associated with chloroplasts (Sastry and Kates, 1964; Helmsing, 1969; Anderson et al., 1974; Matsuda et al., 1979). They act as lipolytic acyl hydrolases (LAH), converting galactosyl diacylglycerols into their monoacyl forms and free fatty acids. The monoacyl forms are then further converted to free fatty acids, glycerol and galactose by galactosyl hydrolases and galactosidases (Sastry and Kates, 1964; El-Hafid et al., 1989). There has been a report that β-galactosidase expression is up-regulated in the latter stages of senescence (King et al., 1995), and it has been inferred that galactolipase activity increases in isolated chloroplasts aged *in vitro* (Wintermans et al., 1969; Saczynska et al., 1994). However, there have been no definitive reports of increased galactolipase accumulation or activity during senescence (Smart, 1994). Therefore, while galactolipase activity is presumed to be associated with senescence, its exact role has not yet been clearly defined (Woolhouse, 1984; Matile, 1992). Similarly, it is assumed that a galactolipase is involved in thylakoid lipid turnover (Douce and Joyard, 1980). However, a direct relationship between the enzyme and turnover has not been established. Galactolipase activity has been correlated with sensitivity to chilling injury in *Zea mays* (Kaniuga et al., 1999). However, it is not clear whether the isozyme that is presumed to be involved in turnover and/or senescence is the same as the chilling-specific

isoform. Furthermore, the specificity of galactolipase(s) has yet to be conclusively determined. That is, there is some evidence to suggest that galactolipid-degrading enzymes are in fact general lipolytic acyl hydrolases (Galliard, 1980). However, the expression of a non-specific LAH during senescence fails to explain the selective depletion of galactolipids seen in leaves during senescence (Fong and Heath, 1977; Tevini, 1976).

A gene corresponding to a galactolipase has not yet been isolated. Cloning of a galactolipase gene would significantly help in elucidating the role that this enzyme plays in thylakoid lipid turnover and senescence.

## 1.7 OBJECTIVES OF RESEARCH

The main objective of the current study was to characterize the lipid particles of *Phaseolus vulgaris* leaf chloroplasts. Specifically, a better understanding of the roles these lipid particles play in chloroplast metabolism was gained by elucidating their composition more fully. Indeed, the research has indicated a possible role for the thylakoid-derived lipid particles in Rubisco holoenzyme assembly, in that they contain Rubisco large subunit as well as cpn60, but no detectable small subunit. Furthermore, the finding that chloroplastic lipid-protein particles contain the plastoglobuli-specific protein, PAP, indicates that these lipid bodies are plastoglobuli-like particles. In addition, evidence is presented that both plastoglobuli and lipid-protein particles contain catabolites of cytochrome *f*, indicating that both types of particle are involved in the removal of such destabilizing protein catabolites from thylakoids during normal membrane turnover. This contention is strengthened by the finding that lipid-protein particles are enriched in lipid catabolites such as free fatty acids. Finally, an effort to characterize metabolic activity associated with the chloroplastic lipid-protein particles and/or

thylakoids began with the isolation of three partial lipase clones from leaves. One of these three partial lipase clones may correspond to a lipase that is up-regulated during leaf senescence, as indicated by Northern blot analysis. It is also possible that one of the clones is targeted to chloroplasts and is involved in chloroplast lipid turnover or senescence. The findings of the study are presented in three chapters and the results are summarized and integrated in a final discussion.

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# 2. Characterization of Thylakoid-Derived Lipid-Protein Particles Bearing the Large Subunit of Ribulose-1,5-Bisphosphate Carboxylase/Oxygenase[1]

## 2.1 INTRODUCTION

Rubisco catalyzes the first reaction in the photosynthetic fixation of carbon dioxide and thus provides an essential link between inorganic and organic carbon (Andrews and Lorimer, 1987; Hartman and Harpel, 1994). This stromal enzyme is a complex hexadecameric polypeptide of about 560 kD, consisting of eight large and eight small subunits (Andrews and Lorimer, 1987). Rubisco large subunit (RLSU) is encoded by plastid DNA (plastome), synthesized on 70S chloroplast ribosomes and has a molecular mass in the range of 50-56 kD (Andrews and Lorimer, 1987; Langridge, 1981; Berry et al., 1985). The nuclear-encoded Rubisco small subunit (RSSU) is synthesized on free, cytoplasmic ribosomes and has a mature molecular mass of 12-15 kD after being imported into the stroma (Andrews and Lorimer, 1987; Langridge, 1981; Chua and Schmidt, 1978).

It has been demonstrated that at least a portion of RLSU synthesis occurs on thylakoid-bound polysomes of spinach and pea chloroplasts (Bhaya and Jagendorf, 1984; Hattori and Margulies, 1986). These polysomes have been implicated in the co-translational insertion of thylakoid-localized proteins encoded by the plastome (Chua et al., 1973; Margulies et al., 1975; Michaels and Margulies, 1975; Minami and Watanabe, 1984; Kim et al., 1991). RLSU is synthesized as a precursor polypeptide, and post-translational modification removes 14 N-

[1]The work presented in this chapter has been published in *Plant Physiology* (1997) 115:1073-1082 with co-authors S. Ghosh, E.B. Dumbroff and J.E. Thompson

terminal amino acids (1-2 kD) to yield mature large subunit that is assembled into Rubisco holoenzyme (Langridge, 1981; Amiri et al., 1984). This cleavable amino-terminal region may contain a thylakoid targeting sequence, which would support the idea that RLSU is associated with thylakoids prior to incorporation into holoenzyme (Hattori and Margulies, 1986; Klein et al., 1988; Makino and Osmond, 1991). Indeed, it has been suggested that free, precursor RLSU is associated with thylakoids (Kirk, 1978; Makino and Osmond, 1991), although a physiological role for the association of unassembled or ribosome-free RLSU with thylakoids has not been established. In particular, it is not clear whether RLSU synthesized by thylakoid-bound polysomes is directly incorporated into native Rubisco or co-translationally inserted into thylakoids and subsequently released into the stroma for holoenzyme assembly. In the stroma, unassembled large subunit monomers are found in association with chaperonin 60 (cpn60), which is required for the light-dependent assembly of Rubisco (Roy, 1989; Gatenby and Ellis, 1990; Ellis and van der Vies, 1991; Hartman and Harpel, 1994). Rubisco holoenzyme has also been found loosely associated with thylakoids, both independently (Mori et al., 1984; Makino and Osmond, 1991) and as part of a functional multienzyme complex (Suss et al., 1993; Suss et al., 1995). However, the exact mechanism for folding and assembly of higher plant Rubisco remains elusive. In particular, it appears that the *in vitro* formation of higher plant hexadecameric Rubisco is constrained by an inability to form the soluble, eight large-subunit core of the enzyme from individual, insoluble large subunits (Gutteridge and Gatenby, 1995).

It has been demonstrated that lipid-protein particles isolated from the stroma of intact chloroplasts or generated *in vitro* from thylakoids contain thylakoid proteins and their catabolites (Ghosh et al., 1994). Lipid-protein particles differ from microvesicles in that they

are surrounded by a monolayer of polar lipid, and they are thought to be formed by blebbing from membranes (Ghosh et al., 1994; Thompson et al., 1997). In this study, it is demonstrated that a subpopulation of chloroplastic lipid-protein particles originating from thylakoids contains the large subunit of Rubisco, but no detectable small subunit. As well, preliminary data suggest that cpn60 is also associated with lipid-protein particles isolated from the stroma. These findings support earlier work indicating that RLSU is associated with thylakoids prior to its assembly into holoenzyme (Hattori and Margulies, 1986; Klein et al., 1988; Makino and Osmond, 1991) and suggest that release of thylakoid-associated large subunit by blebbing may be an early step in the assembly of functional Rubisco.

## 2.2 MATERIALS AND METHODS

### 2.2.1 Plant Material

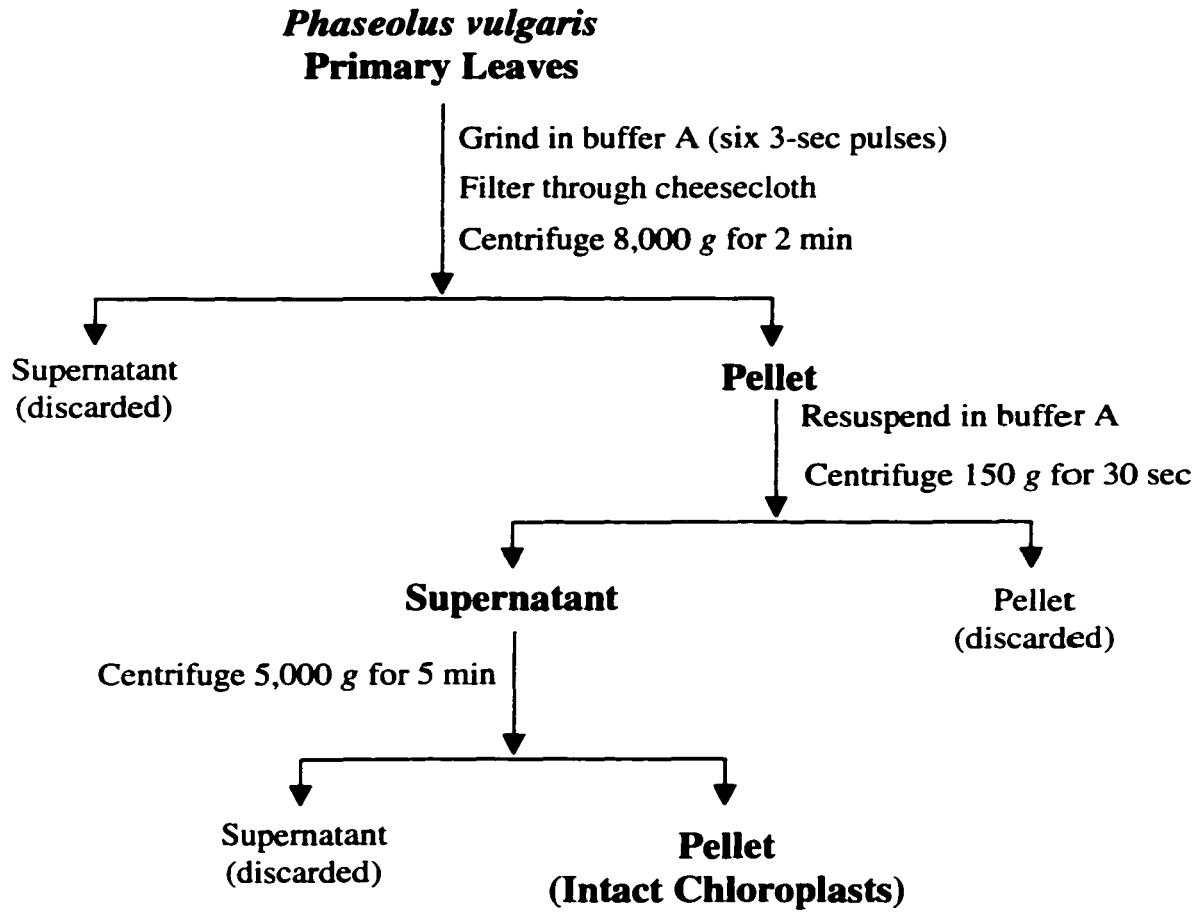
Seedlings of *Phaseolus vulgaris* L. cv Kinghorn wax (yellow wax bean), were grown in flats (54 x 27 x 6 cm) of Pro-mix BX (Premier Brands, Red Hill, PA) under greenhouse conditions with a supplementary 16-h photoperiod of fluorescent light.

### 2.2.2 Subcellular Fractionation

#### *2.2.2.1 Isolation of Intact Chloroplasts and Thylakoids*

Chloroplasts were isolated from the primary leaves of 10-14 day old seedlings, as described by Ghosh et al. (1994). The isolation procedure is illustrated in Figure 2.1. Leaf tissue was homogenized with a Sorvall Omnimixer (six 3-second pulses) in buffer A (5.5 $ml \cdot g^{-1}$) containing 50 mM Epps-KOH (pH 7.8), 10 mM $MgCl_2$, 10 mM $NaHCO_3$, 250 mM D-sorbitol and 1% (v/v)

Figure 2.1. Flow-chart illustrating the isolation of intact chloroplasts from the primary leaves of 10-14-d-old *Phaseolus vulgaris* seedlings.


*Phaseolus vulgaris*
**Primary Leaves**
Grind in buffer A (six 3-sec pulses)
Filter through cheesecloth
Centrifuge 8,000 *g* for 2 min
Supernatant
(discarded)
**Pellet**
Resuspend in buffer A
Centrifuge 150 *g* for 30 sec
**Supernatant**
Pellet
(discarded)
Centrifuge 5,000 *g* for 5 min
Supernatant
(discarded)
**Pellet**
**(Intact Chloroplasts)**

glycerol. The homogenate was filtered through 4 layers of cheesecloth to remove fibrous material, and the filtrate was centrifuged at 8,000 *g* for 2 minutes at 4°C in a GSA rotor (Sorvall). The pellet was resuspended in 300 ml of buffer A and centrifuged at 150 *g* for 30 seconds. The pellet was discarded, and the supernatant was recentrifuged at 5,000 *g* for 5 minutes at 4°C to collect intact chloroplasts in the pellet.

Thylakoids were isolated from intact chloroplasts as previously described (Ghosh et al., 1994) and as outlined in Figure 2.2. The intact chloroplasts were suspended in hypotonic lysis buffer (1 mg chlorophyll·$mL^{-1}$) containing 10 mM Epps-KOH (pH 7.8), 10 mM $MgCl_2$ and 10 mM $NaHCO_3$, and incubated for 30 min on ice in the dark. The lysate was centrifuged at 12,000 *g* for 10 min in a GSA rotor (Sorvall) to yield pelleted thylakoids and a supernatant containing the stroma. The thylakoids were washed by resuspending the pellet in buffer A (1.5 mg chlorophyll·$mL^{-1}$) and centrifuging at 12,000 *g* for 10 min. In some experiments, thylakoids were washed more stringently, by treatment with 0.1 M $Na_2CO_3$ (pH 11.0) or 0.5% (v/v) Triton X-100. Following resuspension in the wash solution thylakoids were collected by centrifugation at 12,000 *g* for 10 min and resuspended in buffer A for further analysis.

#### *2.2.2.2 Isolation of Stromal Lipid-Protein Particles*

The full complement of chloroplast lipid-protein particles can be isolated by ultrafiltration of ultracentrifuged stroma as outlined in Figure 2.2 (Ghosh et al., 1994). In the present study, a subpopulation of lipid-protein particles was isolated from the stroma by flotation centrifugation as outlined in Figure 2.3A. For this purpose, the stroma was made 20% (w/v) with sucrose, overlaid with lysis buffer and centrifuged at 4°C for 12 h at 305,000 *g* in a 60 Ti rotor (Beckman). The upper layer containing the floated lipid-protein particles was removed

Figure 2.2. Flow-chart illustrating the isolation of thylakoids and stromal lipid-protein particles from intact chloroplasts.

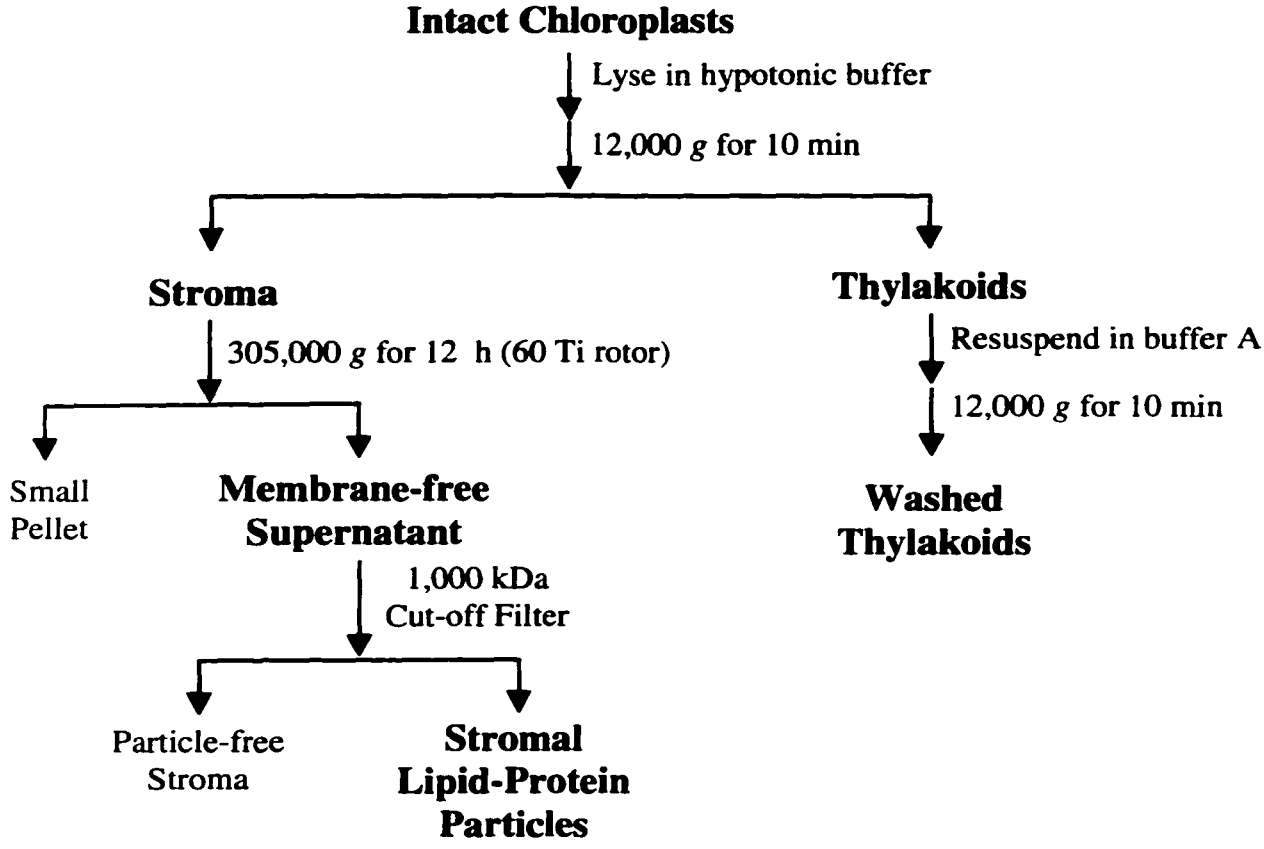
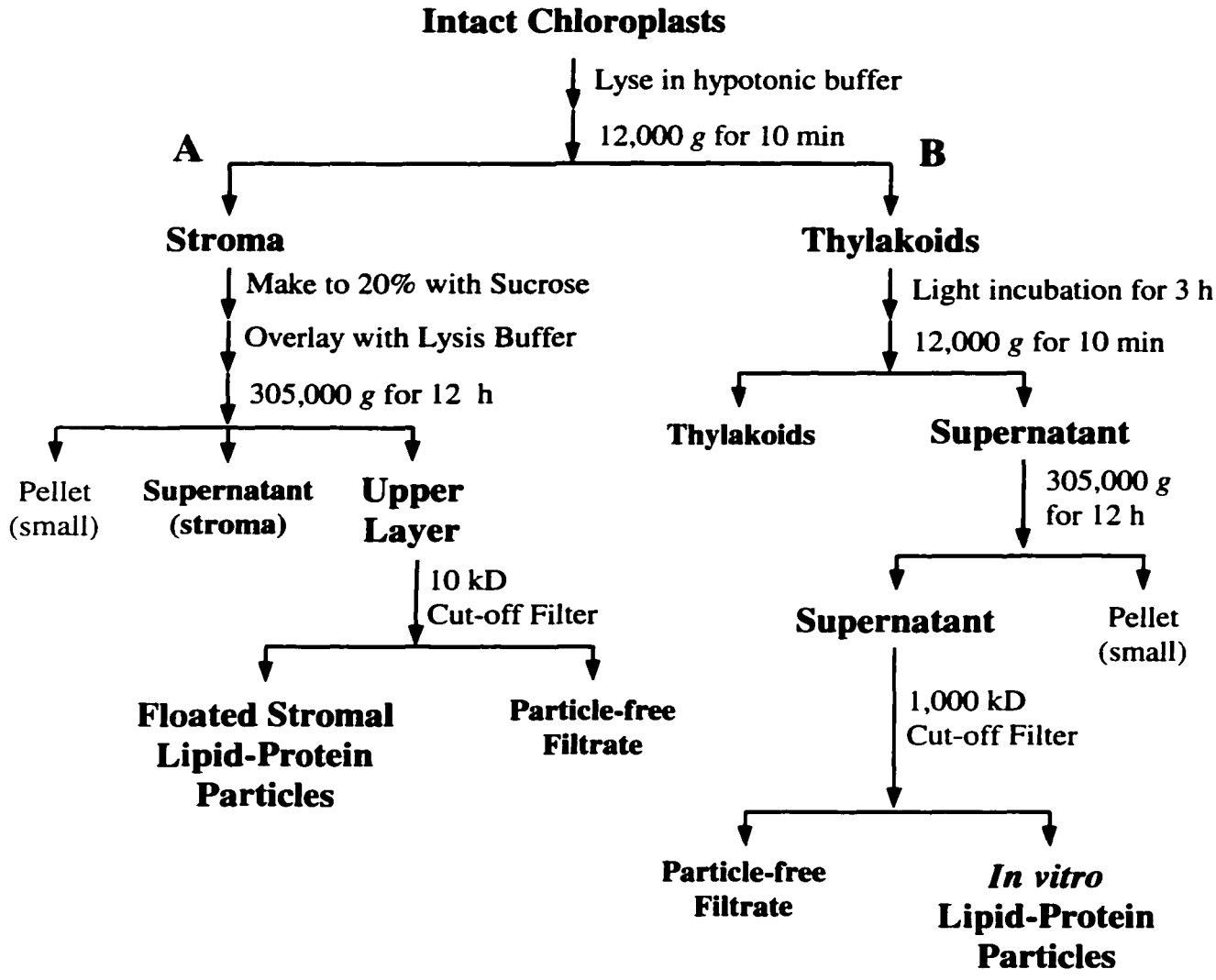

Intact Chloroplasts
Lyse in hypotonic buffer
12,000 g for 10 min
Stroma
Thylakoids
305,000 g for 12 h (60 Ti rotor)
Resuspend in buffer A
12,000 g for 10 min
Small Pellet
Membrane-free Supernatant
Washed Thylakoids
1,000 kDa Cut-off Filter
Particle-free Stroma
Stromal Lipid-Protein Particles

Figure 2.3. Flow-chart illustrating the isolation of a subpopulation of stromal lipid-protein particles by flotation centrifugation (A) and the generation of lipid-protein particles *in vitro* from isolated thylakoids (B).


Intact Chloroplasts
Lyse in hypotonic buffer
12,000 g for 10 min
A
B
Stroma
Thylakoids
Make to 20% with Sucrose
Overlay with Lysis Buffer
305,000 g for 12 h
Light incubation for 3 h
12,000 g for 10 min
Thylakoids
Supernatant
Pellet (small)
Supernatant (stroma)
Upper Layer
10 kD Cut-off Filter
305,000 g for 12 h
Supernatant
Pellet (small)
1,000 kD Cut-off Filter
Floated Stromal Lipid-Protein Particles
Particle-free Filtrate
Particle-free Filtrate
In vitro Lipid-Protein Particles

and concentrated ~20-fold using Centricon-10 filters (Amicon) (Fig. 2.3A). In some experiments, the stroma was passed through a 1,000 kD cut-off filter (Filtron Technology Corp., Northborough, MA) to obtain particle-free stroma.

#### *2.2.2.3* In vitro-*Generation of Lipid-Protein Particles*

Lipid-protein particles were also generated *in vitro* from thylakoids as described by Ghosh et al. (1994) and as outlined in Figure 2.3B. Washed thylakoids were suspended in buffer A (1.5 mg chlorophyll·mL$^{-1}$) and incubated under light (525 μmol·m$^{-2}$·s$^{-1}$), in darkness or in darkness with 5 mM ATP at 21°C for 3 h. In some experiments, thylakoids were heat-denatured by boiling for 5 min prior to being used for *in vitro*-generation of particles. Following the 3 h incubation, the suspension was centrifuged at 12,000 *g* for 10 min to yield a supernatant containing the *in vitro*-generated lipid-protein particles. The supernatant was centrifuged again for 12 h at 305,000 *g* and 4°C to remove any residual membranes. The lipid-protein particles, which remained in suspension, were isolated by ultrafiltration of this supernatant through a 1,000 kD cut-off filter (Filtron) (Fig. 2.3B).

#### *2.2.2.4 Gel Filtration Chromatography*

*In vitro*-generated lipid-protein particles and Rubisco holoenzyme (Sigma) were fractionated by size-exclusion Sephacryl S-300 HR (Pharmacia) chromatography. The column (1.6 cm i.d. x 95 cm) was calibrated using protein standards from Pharmacia. Lipid-protein particle and Rubisco (Sigma) suspensions in buffer A (5 mL containing 500 μg protein) were loaded onto the column and eluted with buffer (50 mM Epps-KOH, pH 7.8, 10 mM $MgCl_2$, 10

mM $NaHCO_3$, 1% (v/v) glycerol) at 4°C. Fractions of 4 mL were collected and assayed for total protein, lipids and Rubisco activity.

#### *2.2.2.5 Immunopurification of Lipid-Protein Particles Containing RLSU*

A subpopulation of chloroplast lipid-protein particles containing RLSU was obtained by immunopurification. For this purpose, an immunoaffinity column (4 mL) of Rubisco holoenzyme antibody crosslinked to protein A-agarose (Sigma) was prepared using dimethylsuberimidate dihydrochloride as the crosslinker as described by Reeves et al. (1981). After washing the column with 0.85% saline (20 mL), 10 mL (~1 mg protein) of *in vitro*-generated lipid-protein particles or 10 mL (~40 µg protein) of floated stromal lipid-protein particles were recirculated through the column for 2 h. The column was then washed with 20 mL of 0.85% saline to remove unbound proteins. The particles containing RLSU were then eluted with 12 mL of 4 M guanidine hydrochloride, desalted and concentrated using Centricon-10 filters (Amicon) and analyzed for lipid and protein. A protease inhibitor cocktail consisting of 2 mM PMSF, 10 mM EDTA and 0.1 mg/mL Pepstatin A was used throughout the immunopurification process.

#### *2.2.2.6 Isolation of Microsomal Membranes and Cytosolic Lipid-Protein Particles*

Microsomal membranes and cytosolic lipid-protein particles were isolated from cotyledons of 2 day-old *Phaseolus vulgaris* seedlings (cv Kinghorn) grown in vermiculite at 27°C under conditions of etiolation. Total microsomal membranes were isolated and washed according to Yao et al. (1991) except that phosphate buffer (50 mM phosphate, pH 7.5, 8.5% (w/v) sucrose, 2 mM EDTA, 5 mM EGTA, 0.1 mM PMSF, 0.1 mM Benzamidine-HCl) was

used. Specifically, the coat was peeled from 100 seeds (200 cotyledons) 2 days after planting. The tissue was homogenized with a Polytron homogenizer (1 min) in 3 volumes of phosphate buffer and filtered through 4 layers of cheesecloth. The filtrate was then centrifuged at 10,000 *g* for 20 min at 4°C in an SS-34 rotor (Sorvall). The pellet was discarded, and the supernatant was centrifuged at 250,000 *g* for 1 h at 2°C in a 60Ti rotor (Beckman) to isolate total microsomal membranes in the pellet. The white, floated layer from this centrifugation, containing oil bodies, was discarded, and the supernatant (post-microsomal supernatant) was retained for isolation of lipid-protein particles. The total microsomal membranes (pellet) were washed by suspension in phosphate buffer and centrifugation at 250,000 *g* for 1 h. Cytosolic lipid-protein particles were isolated by flotation centrifugation as described by Hudak and Thompson (1996). Briefly, cytosol (post-microsomal supernatant) was made 20% (w/v) with sucrose, overlaid with phosphate buffer and centrifuged at 2°C for 16 h at 305,000 *g* in a 60 Ti rotor. The upper layer containing the floated lipid-protein particles was removed and used for further analyses.

#### *2.2.2.6.1 Treatment of Microsomal Membranes and Cytosolic Lipid-Protein Particles with Particle-Free Stroma*

Total washed microsomal membranes isolated from etiolated bean cotyledons were resuspended in freshly isolated chloroplast stroma that had been rendered free of stromal lipid-protein particles by passage through a 1,000 kD cut-off filter (as described in section 2.2.2.2) at a concentration of 10 mg membrane protein per mg of stromal protein. Treated microsomal membranes were then re-isolated by centrifugation for 1 h at 250,000 *g*, and resuspended in a small volume of buffer A for further analyses.

Cytosolic lipid-protein particles isolated from etiolated bean cotyledons were diluted with freshly prepared ultrafiltered stroma to a concentration of 0.1 mg of particle protein per mg of stromal protein. The mixture was then made to 20% (w/v) with sucrose, overlaid with sucrose-free buffer A, and centrifuged for 16 h at 305,000 *g*. The upper floated layer, containing the stroma-treated lipid-protein particles, was removed and retained for further analyses.

### 2.2.3 Protein Analyses

#### *2.2.3.1 Protein Quantitation*

Protein concentration was measured according to Bradford (1976). Up to 100 µL of sample was diluted with buffer A to a final volume of 2.5 mL. The total assay volume was brought to 5 mL by the addition of 2.5 mL of reagent (consisting of 10% (v/v) ethanol, 0.02% (w/v) Coomassie brilliant blue G-250 and 20% (v/v) of 85% $H_3PO_4$, filtered through Whatman #1 paper). The assay mixture was incubated for 10 min at room temperature. Absorbance was measured at 595 nm in a DU-64 spectrophotometer (Beckman) zeroed using assay solution diluted 1:1 with buffer A. Protein was quantified using bovine serum albumin as a standard.

Alternatively, protein concentration was determined according to Ghosh et al. (1988) using bovine serum albumin as a standard. Samples were diluted in an equal volume of SDS-PAGE sample buffer, and 1 µL dots of the mixture were placed on Whatman 3 MM blotting paper. After allowing time for the spots to dry, the blotting paper was incubated in stain (40% (v/v) methanol, 10% (v/v) acetic acid, 0.2% (w/v) Coomassie brilliant blue R-250) for 20 minutes at room temperature, with gentle mixing. The paper was then incubated in several changes of destain (40% (v/v) methanol, 10% (v/v) acetic acid) to remove background stain

from the blotting paper. Protein concentration was determined by densitometry and comparison to a series of BSA standard spots. To accomplish this, an image of the blot was captured using an Hewlett-Packard ScanJet 3p scanner connected to a computer, and the integrated volume of each spot was measured using ImageQuant computer software.

### *2.2.3.2 Polyclonal Antibody Production*

The aligned amino acid sequences of RLSU from five higher plant species are presented in Figure 2.4. The alignment was performed using MultAlin version 5.3.3 (Corpet, 1988), a multiple sequence alignment program available on the internet (W3.toulouse.inra.fr), and illustrates the high degree of homology among Rubisco large subunits. A peptide was synthesized that corresponds to a highly conserved region of RLSU. This peptide ($A_{430} - C_{449}$; $H_2N$-ARNEGRDLAREGNTIIREAC-COOH) is highlighted in Figure 2.4, and was used to generate polyclonal antibodies. This 20-amino acid peptide was selected on the basis of three criteria. First, this portion of RLSU is hydrophilic as determined by hydropathy analysis. The hydropathy plot for RLSU is shown in Figure 2.5 and was generated using the scale of Kyte and Doolittle (1982) available on the ExPASy Molecular Biology Server on the internet (www.expasy.ch). Hydrophilicity correlates positively with antigenicity (Drenckhahn et al., 1993). Secondly, this portion of RLSU is highly conserved across the five higher plants that were compared (Fig. 2.4), which indicates that the corresponding antibody will react with RLSU from a wide range of plants. Finally, the cysteine residue located at the C-terminus of the peptide facilitates conjugation of the peptide through its sulphydryl group, to a carrier

Figure 2.4. Alignment of Rubisco large subunit proteins from five different higher plant species. The highlighted region corresponds to the 20-amino acid synthetic peptide that was used to generate RLSU-specific polyclonal antibodies (ARNEGRDLAREGNTIIREAC). All sequences were deduced from the nucleotide sequences of isolated *rbcL* genes, and were obtained from NCBI databanks (www.ncbi.nlh.gov). The alignment was performed by MultAlin version 5.3.3 (Corpet, 1988), a multiple sequence alignment program available on the world wide web (W3.toulouse.inra.fr). Ps, *Pisum sativum*; Gm, *Glycine max*; So, *Spinacia oleracea*; Nt, *Nicotiana tabacum*; At, *Arabidopsis thaliana*. Amino acid residue numbers are indicated, beginning with the N-terminus.

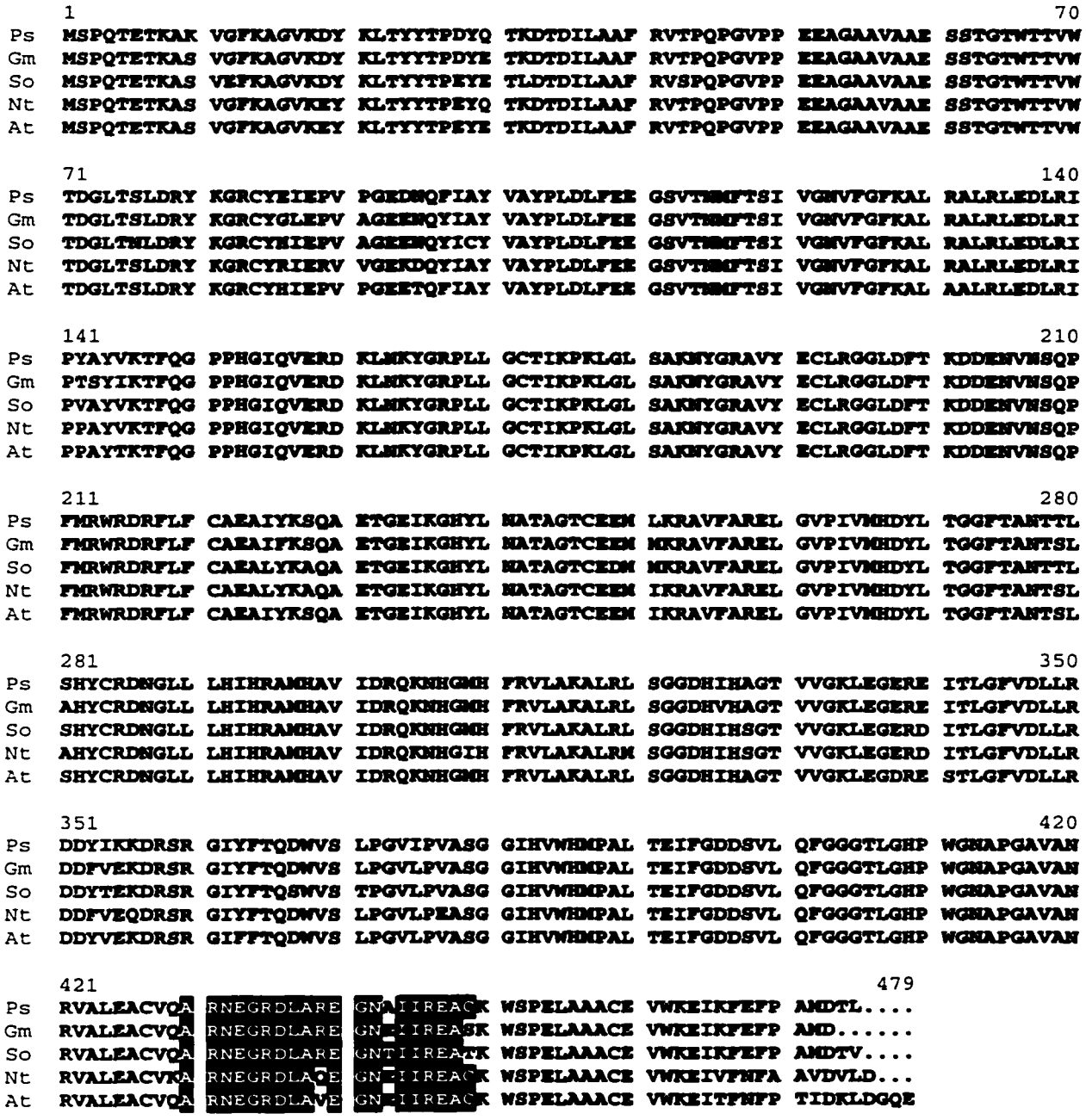
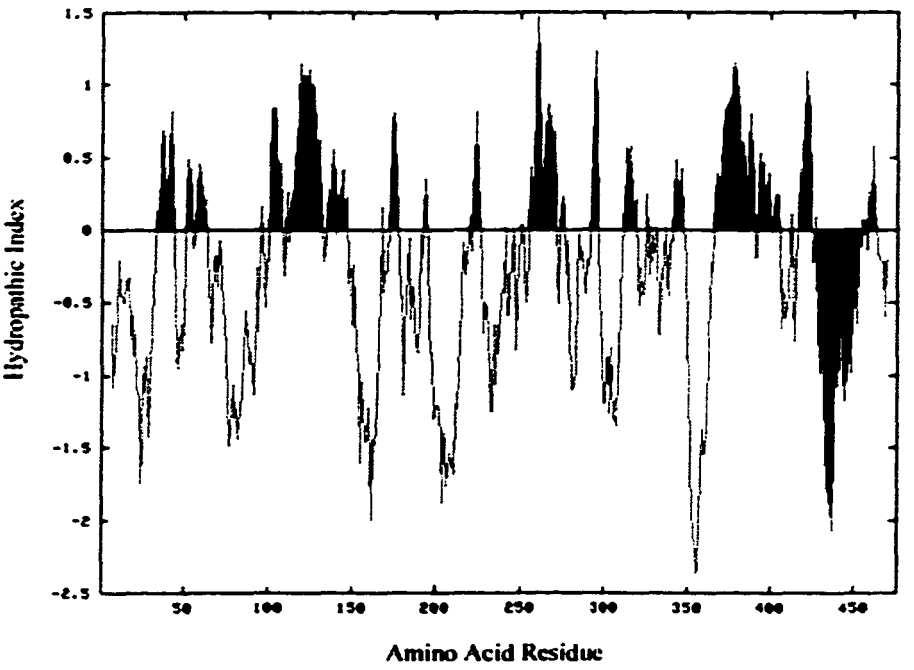

1 70
Ps MSPQTETKAK VGFKAGVKDY KLTYYTPDYQ TKDTDILAAF RVTPQPGVPP EEAGAAVAAE SSTGTWTTVW
Gm MSPQTETKAS VGFKAGVKDY KLTYYTPDYE TKDTDILAAF RVTPQPGVPP EEAGAAVAAE SSTGTWTTVW
So MSPQTETKAS VEFKAGVKDY KLTYYTPEYE TLDTDILAAF RVSPQPGVPP EEAGAAVAAE SSTGTWTTVW
Nt MSPQTETKAS VGFKAGVKEY KLTYYTPEYQ TKDTDILAAF RVTPQPGVPP EEAGAAVAAE SSTGTWTTVW
At MSPQTETKAS VGFKAGVKEY KLTYYTPEYE TKDTDILAAF RVTPQPGVPP EEAGAAVAAE SSTGTWTTVW
71 140
Ps TDGLTSLDRY KGRCYEIEPV PGEDNQFIAY VAYPLDLFEE GSVTNMFTSI VGNVFGFKAL RALRLEDLRI
Gm TDGLTSLDRY KGRCYGLEPV AGEENQYIAY VAYPLDLFEE GSVTNMFTSI VGNVFGFKAL RALRLEDLRI
So TDGLTNLDRY KGRCYNIEPV AGEENQYICY VAYPLDLFEE GSVTNMFTSI VGNVFGFKAL RALRLEDLRI
Nt TDGLTSLDRY KGRCYRIERV VGEKDQYIAY VAYPLDLFEE GSVTNMFTSI VGNVFGFKAL RALRLEDLRI
At TDGLTSLDRY KGRCYHIEPV PGEETQFIAY VAYPLDLFEE GSVTNMFTSI VGNVFGFKAL AALRLEDLRI
141 210
Ps PYAYVKTFQG PPHGIQVERD KLNKYGRPLL GCTIKPKLGL SAKNYGRAVY ECLRGGLDFT KDDENVNSQP
Gm PTSYIKTFQG PPHGIQVERD KLNKYGRPLL GCTIKPKLGL SAKNYGRAVY ECLRGGLDFT KDDENVNSQP
So PVAYVKTFQG PPHGIQVERD KLNKYGRPLL GCTIKPKLGL SAKNYGRAVY ECLRGGLDFT KDDENVNSQP
Nt PPAYVKTFQG PPHGIQVERD KLNKYGRPLL GCTIKPKLGL SAKNYGRAVY ECLRGGLDFT KDDENVNSQP
At PPAYTKTFQG PPHGIQVERD KLNKYGRPLL GCTIKPKLGL SAKNYGRAVY ECLRGGLDFT KDDENVNSQP
211 280
Ps FNRWRDRFLF CAEAIYKSQA ETGEIKGHYL NATAGTCEEM LKRAVFAREL GVPIVMHDYL TGGFTANTTL
Gm FNRWRDRFLF CAEAIFKSQA ETGEIKGHYL NATAGTCEEM MKRAVFAREL GVPIVMHDYL TGGFTANTSL
So FNRWRDRFLF CAEALYKAQA ETGEIKGHYL NATAGTCEDM MKRAVFAREL GVPIVMHDYL TGGFTANTTL
Nt FNRWRDRFLF CAEALYKAQA ETGEIKGHYL NATAGTCEEM IKRAVFAREL GVPIVMHDYL TGGFTANTSL
At FNRWRDRFLF CAEAIYKSQA ETGEIKGHYL NATAGTCEEM IKRAVFAREL GVPIVMHDYL TGGFTANTSL
281 350
Ps SHYCRDNGLL LHIHRAMHAV IDRQKNHGMH FRVLAKALRL SGGDHIHAGT VVGKLEGERE ITLGFVDLLR
Gm AHYCRDNGLL LHIHRAMHAV IDRQKNHGMH FRVLAKALRL SGGDHVHAGT VVGKLEGERE ITLGFVDLLR
So SHYCRDNGLL LHIHRAMHAV IDRQKNHGMH FRVLAKALRL SGGDHIHSGT VVGKLEGERD ITLGFVDLLR
Nt AHYCRDNGLL LHIHRAMHAV IDRQKNHGIH FRVLAKALRM SGGDHIHSGT VVGKLEGERD ITLGFVDLLR
At SHYCRDNGLL LHIHRAMHAV IDRQKNHGMH FRVLAKALRL SGGDHIHAGT VVGKLEGDRE STLGFVDLLR
351 420
Ps DDYIKKDRSR GIYFTQDWVS LPGVIPVASG GIHVWHMPAL TEIFGDDSVL QFGGGTLGHP WGNAPGAVAN
Gm DDFVEKDRSR GIYFTQDWVS LPGVLPVASG GIHVWHMPAL TEIFGDDSVL QFGGGTLGHP WGNAPGAVAN
So DDYTEKDRSR GIYFTQSWVS TPGVLPVASG GIHVWHMPAL TEIFGDDSVL QFGGGTLGHP WGNAPGAVAN
Nt DDFVEQDRSR GIYFTQDWVS LPGVLPEASG GIHVWHMPAL TEIFGDDSVL QFGGGTLGHP WGNAPGAVAN
At DDYVEKDRSR GIFFTQDWVS LPGVLPVASG GIHVWHMPAL TEIFGDDSVL QFGGGTLGHP WGNAPGAVAN
421 479
Ps RVALEACVQA RNEGRDLARE GNEIIREACK WSPELAAACE VWKEIKFEFP AMDTL....
Gm RVALEACVQA RNEGRDLARE GNEIIREASK WSPELAAACE VWKEIKFEFP AMD.....
So RVALEACVQA RNEGRDLARE GNTIIREATK WSPELAAACE VWKEIKFEFP AMDTV....
Nt RVALEACVKA RNEGRDLAQE GNEIIREACK WSPELAAACE VWKEIVFMFA AVDVLD...
At RVALEACVQA RNEGRDLAVE GNEIIREACK WSPELAAACE VWKEITFMFP TIDKLDGQE

Figure 2.5. Hydropathy plot of the amino acid sequence of the large subunit of Rubisco (*Spinacea oleracea*) according to the scale of Kyte and Doolittle (1982) using a window size of 13 amino acids. Light shading indicates hydrophobic domains, and dark shading indicates the hydrophilic domain corresponding to a synthetic peptide that was used to generate polyclonal antibodies. The plot was generated using the ProtScale Tool available on the ExPASy Molecular Biology Server (www.expasy.ch).


Hydropathic Index
1.5
1
0.5
0
-0.5
-1
-1.5
-2
-2.5
50
100
150
200
250
300
350
400
450
Amino Acid Residue

protein. The peptide was synthesized in the laboratory of Dr. G. Lajoie in the Department of Chemistry at the University of Waterloo.

The peptide was conjugated to the carrier protein Keyhole Limpet Hemocyanin (KLH) prior to being used as an antigen for polyclonal antibody production, using the glutaraldehyde conjugation method of Drenckhahn et al. (1993). KLH has approximately 60 surface-exposed lysine residues for facilitating coupling to the peptide. KLH was dissolved in phosphate-buffered saline (PBS; containing [per liter] 80 g NaCl, 2 g KCl, 14.4 g $Na_2HPO_4$ and 2.4 g $KH_2PO_4$, pH 7.4) to a concentration of 1 mg/mL, and an equal amount of peptide (equivalent to 0.3 mM final concentration) was then added to the mixture. An equal volume of 2% (v/v) glutaraldehyde (EM grade) was then added dropwise, with constant stirring, and the solution was mixed gently for 1 h at 4°C. The reaction was stopped by the addition of sodium borohydride to a final concentration of 10 mg/mL and incubation for 1 h at 4°C with gentle mixing. The conjugated peptide solution was then dialyzed overnight against PBS at 4°C. Antibodies were raised in rabbits housed in the animal care facility in the Department of Biology (AUPP# 9561) according to the University of Waterloo's Animal Care Standard Operating Procedures. Freund's complete adjuvant was used for the primary immunization and Freund's incomplete adjuvant was used for subsequent booster injections.

#### *2.2.3.3 SDS-PAGE and Western Blot Analyses*

Samples were dissolved in an equal volume of SDS-PAGE sample buffer consisting of 125 mM Tris-HCl (pH 6.8), 0.2% (v/v) glycerol, 4% (w/v) SDS, 0.1% (v/v) 2-β-mercaptoethanol and 0.0025% (w/v) bromophenol blue. Polypeptides were fractionated by SDS-PAGE in Mini Protean Dual Slab Cells (Bio-Rad) using 1 mm thick 4% acrylamide

stacking gels and 10% or 12% acrylamide separating gels (Laemmli, 1970). The gels were stained with silver (Wray et al., 1981). In some experiments, the fractionated proteins were transferred to nitrocellulose for Western blot analysis according to Dumbroff and Gepstein (1993). Specifically, following separation on 12% gels, proteins were transferred to nitrocellulose paper (0.45 μm pore size, Bio-Rad) in transfer buffer comprised of 25 mM Tris, pH 8.3, 160 mM glycine, 0.002% (w/v) SDS and 20% (v/v) methanol. The wet-transfer was conducted in a Bio-Rad Mini Trans-Blot transfer cell at 4°C for 1 h at 0.4 A using a Bio-Rad model 250/2.5 power supply. Prestained molecular weight standards (Bio-Rad) were used to confirm successful transfer. Following transfer, the protein blots were incubated for 1 h with gentle shaking in Tris-buffered saline (TBS; 25 mM Tris, pH 7.5, 140 mM NaCl) containing 1% (w/v) bovine serum albumin (BSA). Primary antibody was then added, and the blot was incubated overnight at room temperature with gentle shaking.

Selected samples were probed for Rubisco on Western blots using monospecific polyclonal antibody for Rubisco holoenzyme diluted 1:10,000 final concentration (Ghosh et al., 1994), or polyclonal antibody raised against a synthetic peptide corresponding to a conserved, hydrophilic region of RLSU (ARNEGRDLAREGNTIIREAC) diluted 1:1000 final concentration. Western blots were also probed with polyclonal antibody raised against cpn60 (a gift from N. Bate and S. Rothstein, University of Guelph) diluted 1:5000 final concentration. Following two 10-min washes with TBS containing 1% BSA and one 10-min wash with TBS, the blots were treated with goat anti-rabbit IgG secondary antibody conjugated to alkaline phosphatase diluted 1:2500 in TBS for 1 h. Following this incubation, the blots were washed for 10 min once in TBS, once in TBS containing 1% (v/v) Triton X-100 and 5 mM EDTA and then again in TBS. Secondary antibody that had bound to primary

antibody was then visualized by incubating the blot in 10 mL of alkaline phosphatase buffer (100 mM Tris, pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$) containing 66 µL nitroblue tetrazolium (50 mg/mL in 70% (v/v) dimethyl formamide) and 33 µL 3-bromo-4-chloro-indolyl phosphate (50 mg/mL in dimethyl formamide) until purplish-brown bands could be seen. The reaction was stopped by rinsing the blots in distilled water.

#### *2.2.3.4 Proteinase K Digests*

In some experiments, lipid-protein particles and thylakoids were treated with proteinase K (1 or 10 $\mu g \cdot mL^{-1}$) at 37°C for up to 120 min prior to Western blot analysis. Digestion was terminated by the addition of phenylmethylsulfonyl fluoride (PMSF) to a final concentration of 10 mM to aliquots taken at various time points.

### 2.2.4 Lipid Analysis

#### *2.2.4.1 Lipid Extraction and Thin Layer Chromatography*

Lipids were isolated essentially as described by Bligh and Dyer (1959). Total lipids were extracted by vigorous mixing after the addition of 3 volumes of chloroform : methanol (1:2 v/v) to the sample. One microgram each of unesterified 17:0 (heptadecanoic acid) and esterified 17:0 (phosphatidylcholine) purchased from Sigma were added as internal standards for every 10 micrograms of protein in the sample. After extraction, one volume of chloroform and 0.8 volumes of 0.73% (w/v) NaCl were added and the suspension was mixed well and the layers allowed to separate overnight.

The bottom chloroform layer containing the lipid was removed using a 9-inch pasteur pipet and transferred to a small, round-bottomed flask. The chloroform was then evaporated at

37°C using a Buchi R110 rotary evaporator. The lipid was dissolved in a few milliliters of chloroform : methanol (6:1 v/v) and transferred to a small glass vial.

The lipid samples in glass vials were dried down under $N_2$ and redissolved in 50 µL chloroform : methanol (6:1). Fifty µL samples were spotted in lanes on silica gel 60 thin layer chromatography (TLC) plates (Mandel Scientific, Guelph, Ontario), and the lipids were fractionated by running plates in a neutral lipid solvent (70 mL petroleum ether, 30 mL diethyl ether and 2 mL acetic acid). The separated lipids were visualized with iodine vapour and identified using authentic standards (monogalactosyl diacyglycerol, diagalactosyl diacylglycerol, diacylglycerol, unesterified oleic acid and linoleyl alcohol, all purchased from Sigma). Free and esterified fatty acids were scraped off the TLC plates separately onto glassine paper using a clean razor blade, and the silica was then poured into test tubes. The lipids were extracted from the silica by adding 2.5 mL of methanol : chloroform : water (2:1:0.8) to each tube, mixing well and centrifuging briefly with a table top centrifuge to pellet the silica. The solvent was then removed to a new vial using a pasteur pipette, and the silica wash was repeated using 2.5 mL methanol : chloroform : water (2:1:0.8). The solvent from this wash was added to that of the first wash, along with 1.5 mL chloroform and 2 mL $ddH_2O$. The suspension was mixed well and allowed to separate for 12 hours. The bottom chloroform phase was retained for analysis of acyl chains.

#### *2.2.4.2 Acyl Chain Quantitation*

The lipid samples were transmethylated according to Morrison and Smith (1964). To this end, the bottom chloroform phase containing the lipids extracted from silica was transferred to a new small glass vial and dried down under $N_2$. One mL of $BF_3/CH_3OH$ was added to each

vial. The vials were sealed under nitrogen, mixed well and incubated at 90°C for 1 hour. After cooling, 1 mL of hexane and 0.5 mL dd$H_2O$ were added. The suspension was mixed well and centrifuged in a table top centrifuge for 5 minutes to separate the layers. The top hexane phase was transferred to a new vial. A second hexane extraction was performed using another 1 mL of hexane. The hexane samples were sealed under nitrogen and stored at -20°C.

In preparation for gas chromatography, the methylated samples were dried down under $N_2$ and redissolved in 10 µL of hexane. The vials were mixed well, and a 1 µL aliquot was injected into a Hewlett-Packard 5890 Series II Gas Chromatograph equipped with a 15 m x 0.25 mm i.d. fused silica capillary column coated with dimethylpolysiloxane containing 50% cyanopropyl substitution (DB-23) (J&W Scientific, Brockville, ON) (the oven temperature was 180°C, the injector temperature 250°C and the detector temperature 275°C). The helium head pressure was maintained at 25 kPa. Retention times and microgram amounts of separated fatty acid methyl esters were recorded on a Hewlett-Packard integrator, and the methyl esters were identified by comparison with authentic standards.

### 2.2.5 Biochemical Analyses

#### *2.2.5.1 Rubisco Activity Measurements*

Rubisco activity was assayed in 1.5 mL microfuge tubes as described by Ghosh et al. (1989). The reaction mixture contained 12.5 µL of $Na^{14}CO_2$ and 12.5 µL of 12 mM RuBP. Up to 100 µL of buffer A (see section 2.2.2.1) and at least 100 µL of sample were then added to each tube to give a final reaction volume of 250 µL (activity could be maximized by adding more sample and less buffer A). The reaction mixture was vortexed well and incubated at room temperature for 10 minutes. The reaction was then terminated by the addition of 250 µL

of 6 M acetic acid. The reaction tubes were mixed well and left open for approximately 12 hours in a fume hood designated for working with radioactivity to allow removal of any unreacted $^{14}CO_2$. The reacted mixtures were then transferred to small screw-cap scintillation vials. Three milliliters of EcoLite scintillation cocktail (ICN) was added to each vial, and radioactivity was counted in a Beckman LS7000 scintillation counter. Background levels were measured by substituting 12.5 µL of buffer A for substrate (RuBP). All samples were assayed in duplicate.

#### *2.2.5.2 Chlorophyll Measurements*

Chlorophyll was extracted and quantified as described by Porra et al. (1989). The pigment was extracted by mixing 100 µL of sample with 4.9 mL of dimethyl sulphoxide (DMSO), to give a final assay volume of 5 mL. Absorbances were measured at 647 nm and 664 nm using a Beckman DU-64 spectrophotometer. Total chlorophyll concentration was calculated using the following formula:

$$\text{Chlorophyll a + b } (\mu g/mL) = [(17.67 \times A_{647}) + (7.12 \times A_{664})] \times \text{Dil. Factor}$$

### 2.2.6 Electron Microscopy

Isolated lipid-protein particles were examined by transmission electron microscopy. For this purpose, a droplet of freshly prepared lipid-protein particles was placed on a formvar-coated copper grid. After 1 min, the excess moisture was absorbed using filter paper, and the grid was positively stained for 1 min with uranyl acetate-saturated 70% ethanol and air-dried. The samples were examined using a Phillips 300 transmission electron microscope operating at 60 kV.

### 2.2.7 Light-Scattering Measurements

Isolated lipid-protein particles were sized by dynamic light-scattering using a helium-neon laser (model 125, Spectra Physics, San Jose, CA) as described by Hallett et al. (1989). J. Marsh kindly conducted the analysis at the University of Guelph, Department of Physics.

## 2.3 RESULTS

### 2.3.1 Association of RLSU with Stromal and *In vitro*-Generated Lipid-Protein Particles

Lipid-protein particles were generated *in vitro* by incubating washed thylakoids obtained from intact chloroplasts in buffer under light. Table 2.1 shows that blebbing was inhibited by ~70% when the thylakoid membranes were heat-denatured prior to incubation in light, and by ~50% when the incubation was carried out in darkness. The inhibitory effect of darkness was partially alleviated by the addition of 5 mM ATP to the incubation mixture (Table 2.1).

Lipid-protein particles were also isolated from chloroplast stroma by flotation centrifugation. As shown in Figure 2.6, the protein profiles of *in vitro*-generated lipid-protein particles and those isolated from the stroma are not identical, but they are both clearly distinguishable from the corresponding protein profile for thylakoids. The protein content of the floated stromal lipid-protein particles was underestimated due to the dilute nature of the sample (Fig. 2.6, lane 4). As well, there were only minor differences between the protein profiles of *in vitro* light- and dark-generated lipid-protein particles (Fig. 2.6, lanes 2 and 3). The Western blot in Figure 2.7A (lanes 1 and 2), which was probed with antibodies against Rubisco holoenzyme, indicates that both the *in vitro*-generated and stromal lipid-protein

**Table 2.1.** Effect of heat denaturation, light and ATP on the *in vitro*-generation of lipid-protein particles from thylakoids

| Thylakoid Incubation Conditions | Protein Content[a] ($\mu g \cdot mg\ chlorophyll^{-1}$) |
|---|---|
| Light | 8.8 |
| Darkness | 4.5 |
| Darkness + 5 mM ATP | 7.3 |
| Heat denatured in light | 2.8 |
| Heat denatured in darkness | 1.1 |

[a]Values represent levels of protein in the lipid-protein particles formed during a 3-hour incubation of thylakoids and are expressed relative to thylakoid chlorophyll levels before incubation. Results from one experiment, which is representative of three separate experiments, are shown.

Figure 2.6. SDS-PAGE of thylakoids and lipid-protein particles. Each lane was loaded with 1 μg protein, and the gels were stained with silver. Molecular weight markers (kD) are indicated. Lane 1, thylakoids (12% gel); lane 2, *in vitro* light-generated lipid-protein particles (12% gel); lane 3, *in vitro* dark-generated lipid-protein particles (12% gel); lane 4, stromal lipid-protein particles isolated by flotation centrifugation (10% gel).

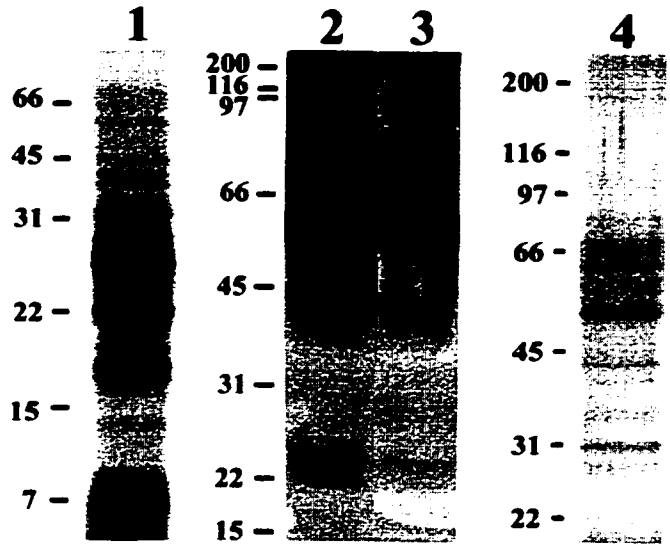
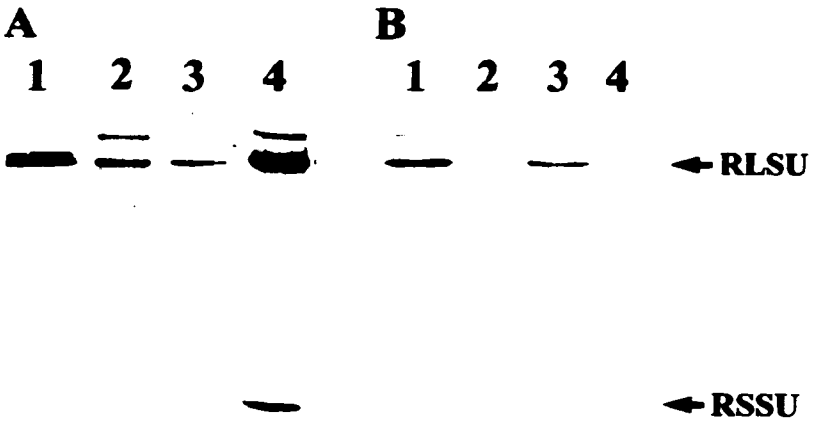

1
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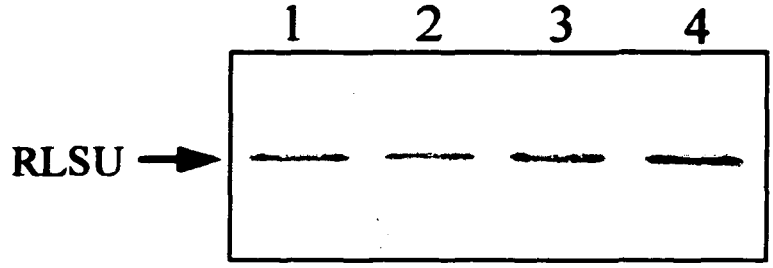
Figure 2.7. Western blots illustrating the association of Rubisco large subunit (RLSU) with lipid-protein particles. Immunodetection was achieved using a monospecific polyclonal antibody against Rubisco holoenzyme. (A): Lane 1, *in vitro* light-generated lipid-protein particles; lane 2, stromal lipid-protein particles isolated by flotation centrifugation; lane 3, thylakoids; lane 4, stroma. The lanes were loaded with equal protein (1 μg). (B): Lane 1, *in vitro* light-generated lipid-protein particles; lane 2, particle-free filtrate obtained by ultrafiltration of the *in vitro* light-generated lipid-protein particles; lane 3, *in vitro* dark-generated lipid-protein particles; lane 4, particle-free filtrate obtained by ultrafiltration of the *in vitro* dark-generated lipid-protein particles. The lanes were loaded with equal volume (35 μL).


A
1 2 3 4
B
1 2 3 4
RLSU
RSSU

particle fractions contain Rubisco large subunit but no detectable small subunit. Indeed, RLSU was substantially enriched in the *in vitro*-generated lipid-protein particles as compared to thylakoids (Fig. 2.7A, lanes 1 and 3). In order to confirm that commensurate levels of RSSU would have been detected under these conditions, Western blots of SDS-PAGE-resolved stroma were run in parallel (Fig. 2.7A, lane 4). As well, Western blot analysis of stroma confirmed that the Rubisco holoenzyme antibody used for this study reacts with both the large and small subunits of *Phaseolus vulgaris* Rubisco (Fig. 2.7A, lane 4). Further evidence supporting the fact that RLSU present in the floated lipid-protein particle fraction is not attributable to Rubisco holoenzyme is the finding that the centrifugation conditions used to float stromal lipid-protein particles sediment Rubisco holoenzyme (Bond, 1995). Figure 2.7B (lanes 1 and 3) demonstrates that more RLSU-containing lipid-protein particles were released from illuminated thylakoids than from those incubated in darkness. Densitometric analysis of the immunodecorated bands in Figure 2.7B indicated that the release of RLSU-containing particles in the dark was only ~40% of that in the light. Large subunit was not detectable in the particle-free filtrate obtained after ultrafiltration of the *in vitro*-generated lipid-protein particles (Fig. 2.7B, lanes 2 and 4).

In order to confirm that RLSU was not simply non-specifically adsorbed to the thylakoid surface, the membranes were treated with 0.1 M $Na_2CO_3$ (pH 11) and 0.5% Triton X-100. Western blot analysis revealed that neither treatment was successful in removing RLSU from thylakoids as compared to crude thylakoids or those washed with buffer A (Fig. 2.8). The exact nature of the association cannot be determined from this analysis, but it does demonstrate that there is a strong interaction between the thylakoids and RLSU.

Figure 2.8. Western blot illustrating the specificity of the association of RLSU with thylakoids. Lane 1, crude thylakoids isolated by centrifugation of lysed chloroplasts; lane 2, thylakoids washed by resuspension in buffer A; lane 3, thylakoids treated with 0.1 M $Na_2CO_3$ (pH 11.0); lane 4, thylakoids treated with 0.5% Triton X-100. Lanes were loaded with equal protein (1 μg), and the blot was probed with Rubisco holoenzyme antibody.


1
2
3
4
RLSU

### 2.3.2 Release of RLSU from Thylakoids by Blebbing of Lipid-Protein Particles

In order to establish that RLSU is released from thylakoids in association with particles and not as a free protein, *in vitro*-generated lipid-protein particles were fractionated on a calibrated Sephacryl S-300 HR size-exclusion column. The protein complement of the lipid-protein particle fraction eluted as a broad peak, but Western blot analysis of the eluted fractions indicated that RLSU was localized in fractions 25-28 corresponding to a molecular mass of ~550 kD (Fig. 2.9, A and B). When Rubisco holoenzyme (Sigma) was fractionated on the same column, most of the protein eluted as a peak in fractions 24-29 coinciding with Rubisco activity, and Western blot analysis confirmed that this peak contained both RLSU and RSSU (Fig. 2.10, A and B). However, when Rubisco was dissociated by treatment with 8 M urea, RLSU and RSSU were resolved by Sephacryl chromatography (Fig. 2.10A). Indeed, Western blot analysis confirmed that free RLSU polypeptide eluted in fractions 30-33 corresponding to its expected molecular weight of ~55 kD (Fig. 2.10C). These observations are consistent with the contention that the large subunit of Rubisco is released from thylakoids as a lipid-protein particle rather than as a free polypeptide.

### 2.3.3 Morphological Features of Lipid-Protein Particles

Light-scattering measurements and electron microscopy further supported the finding that lipid-protein particles are released from thylakoids. The electron micrograph in Figure 2.11 of Sephacryl-resolved fractions bearing RLSU released from thylakoids (fractions 25-28 of Figure 2.9A) illustrates an abundance of individual and clumped spherical particles ranging from 0.05 to 1.0 μm in radius. Light-scattering measurements of the full complement of *in vitro* light-generated lipid-protein particles indicated that the majority of particles are between

Figure 2.9. Fractionation of *in vitro* light-generated lipid-protein particles by Sephacryl chromatography. (A), Protein profile. ●, protein content of eluted lipid-protein particles ($\mu g \cdot fraction^{-1}$); □, molecular weight standards (kD). (B), Immunodetection of RLSU in the eluted fractions with antibody against Rubisco holoenzyme. Each lane was loaded with equal volume (40 µl). Lane 1, fractions 22 and 23; lane 2, fractions 24 and 25; lane 3, fractions 26 and 27; lane 4, fractions 28 and 29; lane 5, fractions 30 and 31; lane 6, fractions 32 and 33; lane 7, fractions 34 and 35.

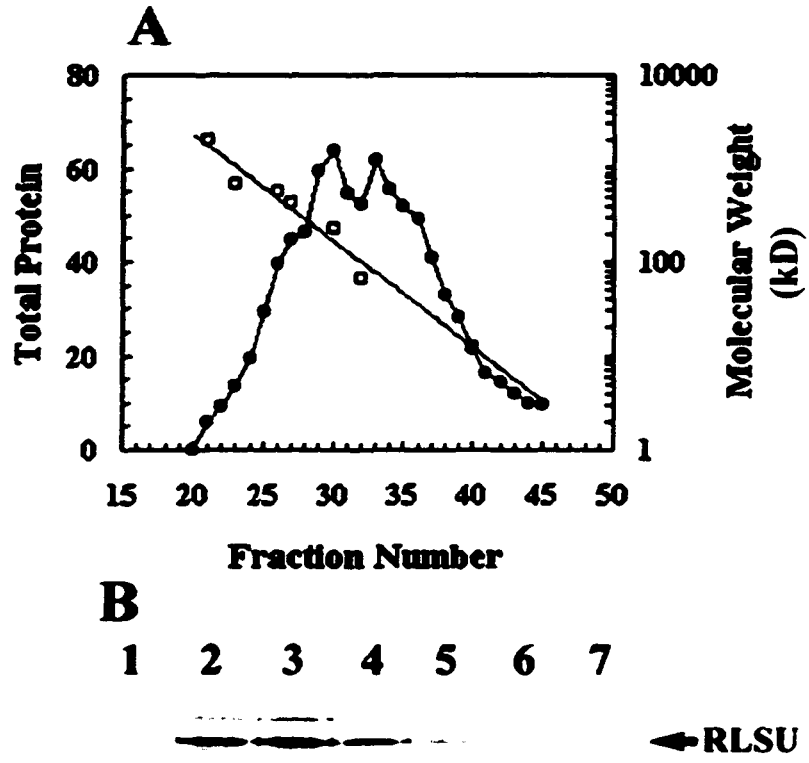
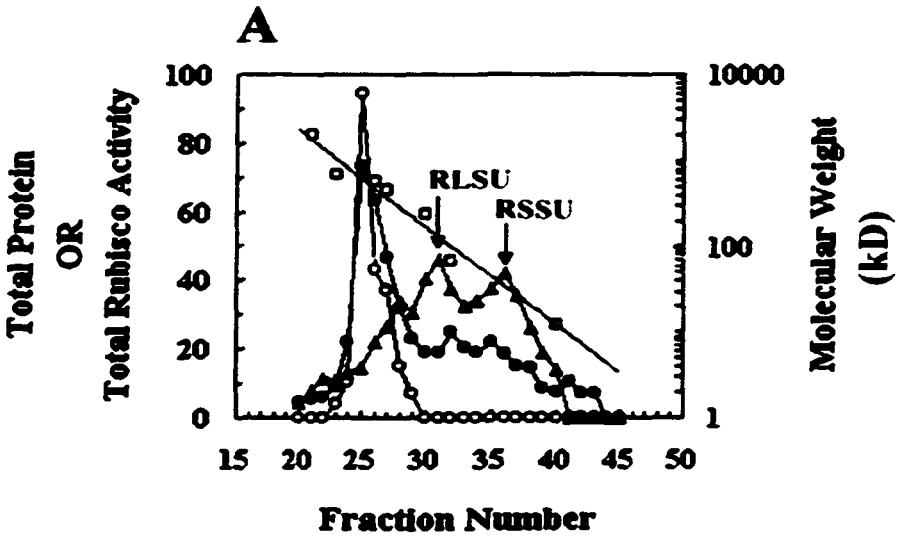
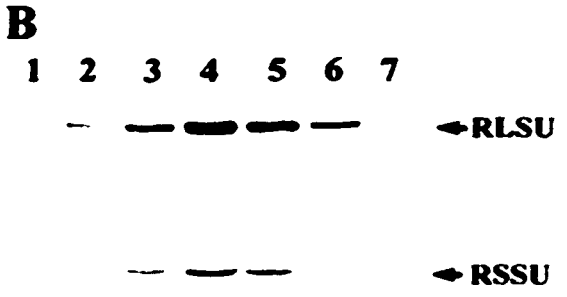

A
80
60
40
20
0
Total Protein
10000
100
1
Molecular Weight (kD)
15 20 25 30 35 40 45 50
Fraction Number
B
1 2 3 4 5 6 7
RLSU

Figure 2.10. Fractionation of Rubisco holoenzyme (purchased from Sigma) by Sephacryl chromatography. (A), 8 M urea-treated Rubisco holoenzyme. ●, protein content of untreated Rubisco fractions (μg·fraction$^{-1}$); ○, activity profile of untreated Rubisco (dpm x $10^{-1}$·fraction$^{-1}$); ▲, protein content of urea-treated Rubisco fractions showing separated subunits (μg·fraction$^{-1}$); □, molecular weight standards (kD). (B), Immunodetection of RLSU and RSSU eluted during fractionation of Rubisco holoenzyme by Sephacryl chromatography. Each lane was loaded with equal volume (40 μL). Lane 1, fractions 20 and 21; lane 2, fractions 22 and 23; lane 3, fractions 24 and 25; lane 4, fractions 26 and 27; lane 5, fractions 28 and 29; lane 6 fractions 30 and 31; lane 7, fractions 32 and 33. (C), Immunodetection of RLSU and RSSU eluted during fractionation of urea-treated Rubisco holoenzyme by Sephacryl chromatography. Each lane was loaded with equal volume (40 μL). Lane 1, fractions 24 and 25; lane 2, fractions 26 and 27; lane 3, fractions 28 and 29; lane 4, fractions 30 and 31; lane 5, fractions 32 and 33; lane 6, fractions 34 and 35; lane 7, fractions 36 and 37; lane 8, fractions 38 and 39; lane 9, fractions 40 and 41.


A
Total Protein
OR
Total Rubisco Activity
100
80
60
40
20
0
10000
100
1
Molecular Weight
(kD)
RLSU
RSSU
15 20 25 30 35 40 45 50
Fraction Number


B
1 2 3 4 5 6 7
RLSU
RSSU

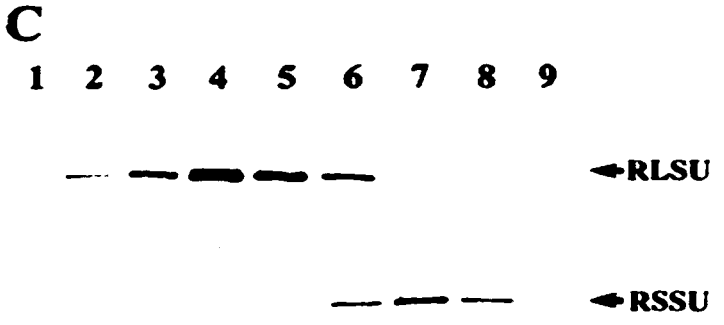
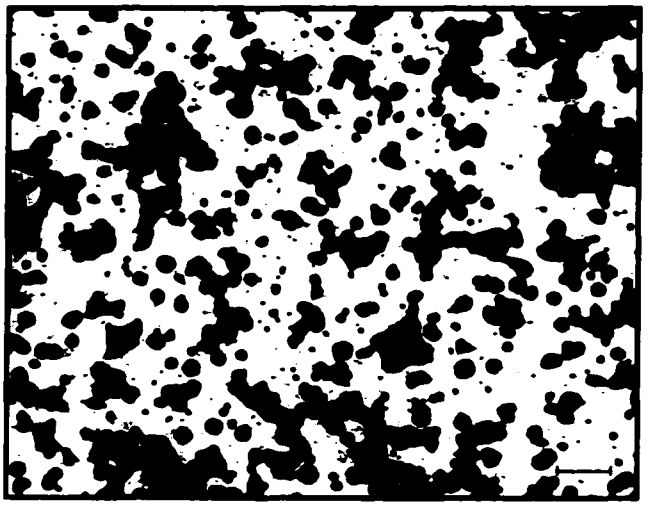

C
1 2 3 4 5 6 7 8 9
RLSU
RSSU

Figure 2.11. Transmission electron micrograph of *in vitro* light-generated lipid-protein particles. Pooled fractions 25-28 from Figure 2.9A containing RLSU are shown. Bar equals 250 nm.



.04 and .07 μm in radius (Figure 2.12). Ghosh et al. (1994) demonstrated previously that stromal lipid-protein particles are also spherical and of a similar size, and are present in chloroplasts *in situ* as well as in isolated stroma.

### 2.3.4 Immunopurification of RLSU-Containing Lipid-Protein Particles

Further confirmation of the contention that RLSU is associated with lipid-protein particles was obtained by immunopurification with Rubisco holoenzyme antibody. Lipid-protein particles bearing large subunit were immunopurified from the population of particles formed *in vitro* from thylakoids and also from the population of *in situ* particles isolated from the stroma by flotation centrifugation. The Western blot analysis of immunopurified particles shown in Figure 2.13 (lanes 1 and 2) confirmed that in both cases RLSU was present. Rubisco small subunit was normally not detected in the immunoprecipitated lipid-protein particles (Fig. 2.13, lanes 1 and 2), although in a few experiments trace amounts were noted for the stromal particles. This presumably reflects contamination of the particles with small amounts of stromal Rubisco. It was apparent as well from SDS-PAGE that proteins in addition to RLSU were present in the particles immunoprecipitated with Rubisco holoenzyme antibody (Fig. 2.13, lanes 3 and 4).

The immunopurified particles also contained lipid. Indeed, as shown in Figure 2.14, all of the fatty acids present in thylakoids were present in both *in vitro* and stromal immunopurified particles, although there was much less 18:3 and relatively more 18:0 in all populations of lipid-protein particles than in thylakoids. Table 2.2 shows that the free to esterified fatty acid ratios of the immunopurified *in vitro* and stromal lipid-protein particles were 0.67 and 1.08, respectively, compared to a corresponding ratio of only 0.07 for

Figure 2.12. Size distribution (radius) of *in vitro* light-generated lipid-protein particles as determined by dynamic light-scattering measurements.

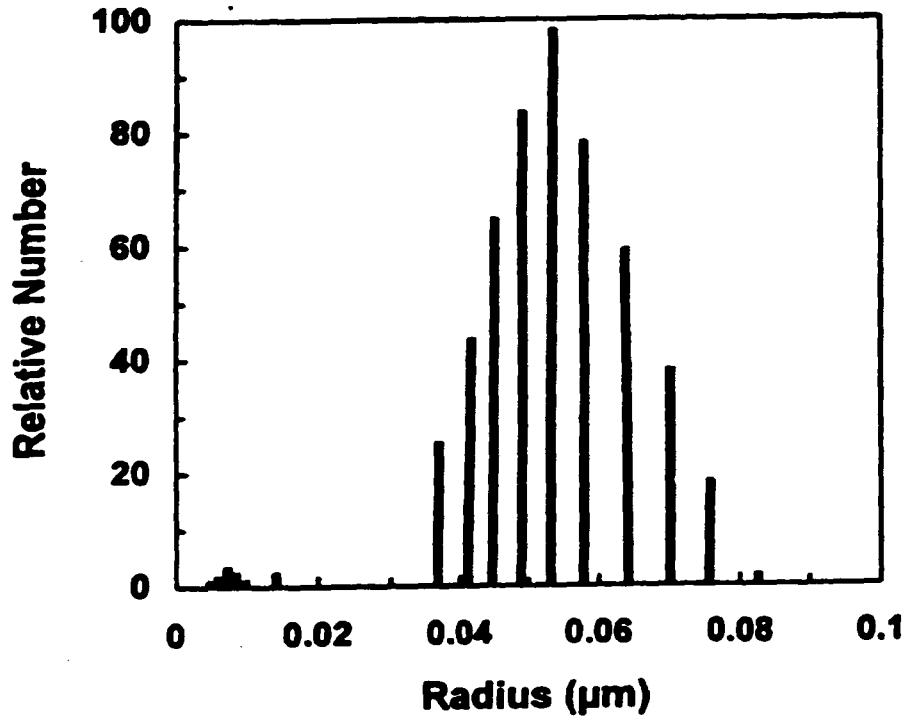

100
80
60
40
20
0
Relative Number
0
0.02
0.04
0.06
0.08
0.1
Radius (μm)

Figure 2.13. SDS-PAGE and Western blots of immunoprecipitated lipid-protein particles. Lane 1, Western blot of immunoprecipitated *in vitro*-generated lipid-protein particles probed with antibody raised against Rubisco holoenzyme; lane 2, Western blot of immunoprecipitated stromal lipid-protein particles probed with antibody raised against Rubisco holoenzyme; lane 3, Silver-stained SDS-PAGE of immunoprecipitated *in vitro*-generated lipid-protein particles; lane 4, Silver-stained SDS-PAGE of immunoprecipitated stromal lipid-protein particles. The lanes were loaded with equal protein (1 μg). Molecular weight markers (kD) are indicated on the right.

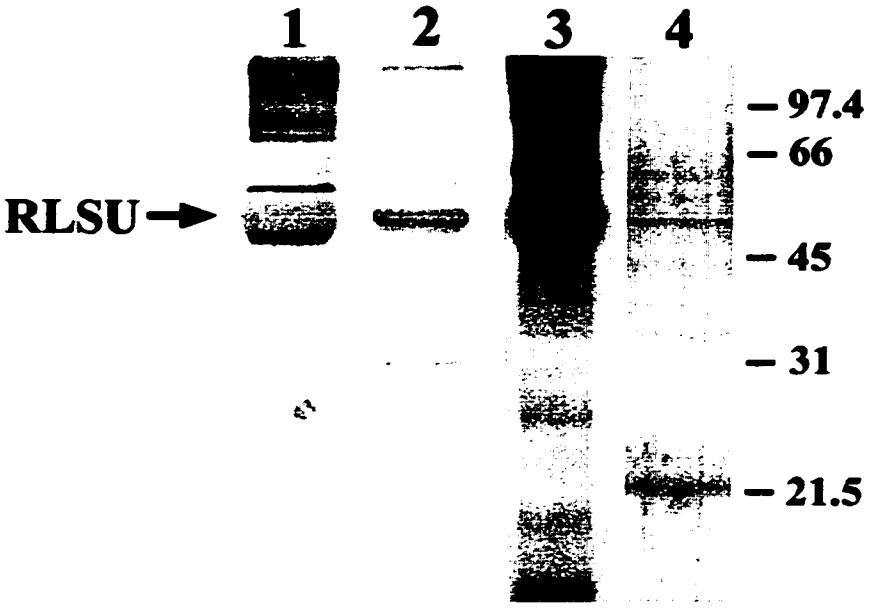

1
2
3
4
RLSU
97.4
66
45
31
21.5

Figure 2.14. Fatty acid composition of thylakoids and lipid-protein particles. Values are expressed as means ± SE for n=3. 16:0, palmitic acid; 16:1, palmitoleic acid; 18:0, stearic acid; 18:1, oleic acid; 18:2, linoleic acid; 18:3, linolenic acid. LPPs, lipid-protein particles.

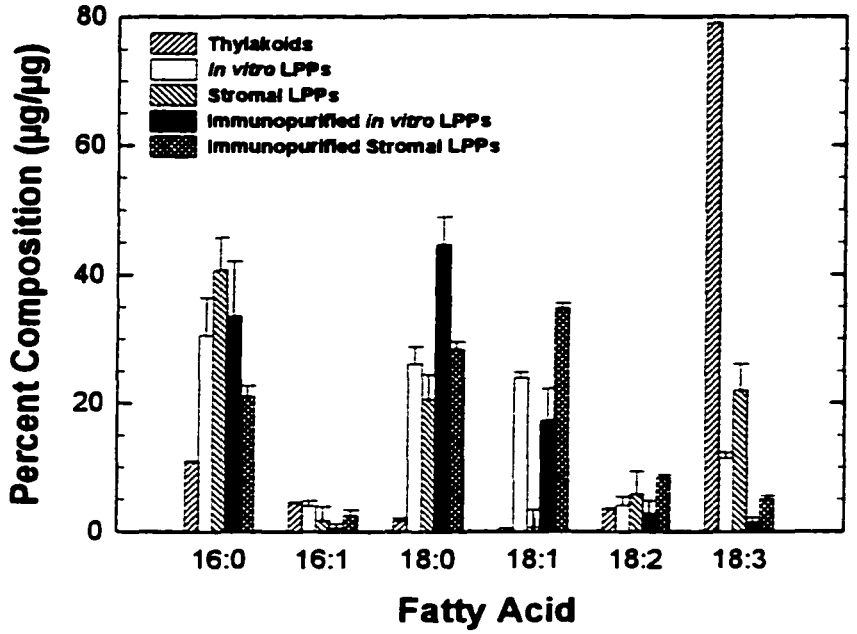

Thylakoids
In vitro LPPs
Stromal LPPs
Immunopurified in vitro LPPs
Immunopurified Stromal LPPs
Percent Composition (µg/µg)
0
20
40
60
80
16:0
16:1
18:0
18:1
18:2
18:3
Fatty Acid

**Table 2.2.** Free and esterified fatty acid levels in thylakoids and lipid-protein particles

| Sample | Free : Esterified Fatty Acid Ratio[a] (μg/μg) |
|---|---|
| Thylakoids | 0.07 ± 0.004 |
| *In vitro* light-generated lipid-protein particles | 0.60 ± 0.07 |
| Stromal lipid-protein particles isolated by flotation centrifugation | 0.71 ± 0.16 |
| Immunopurified *in vitro* light-generated lipid-protein particles | 0.67 ± 0.30 |
| Immunopurified stromal lipid-protein particles | 1.08 ± 0.19 |

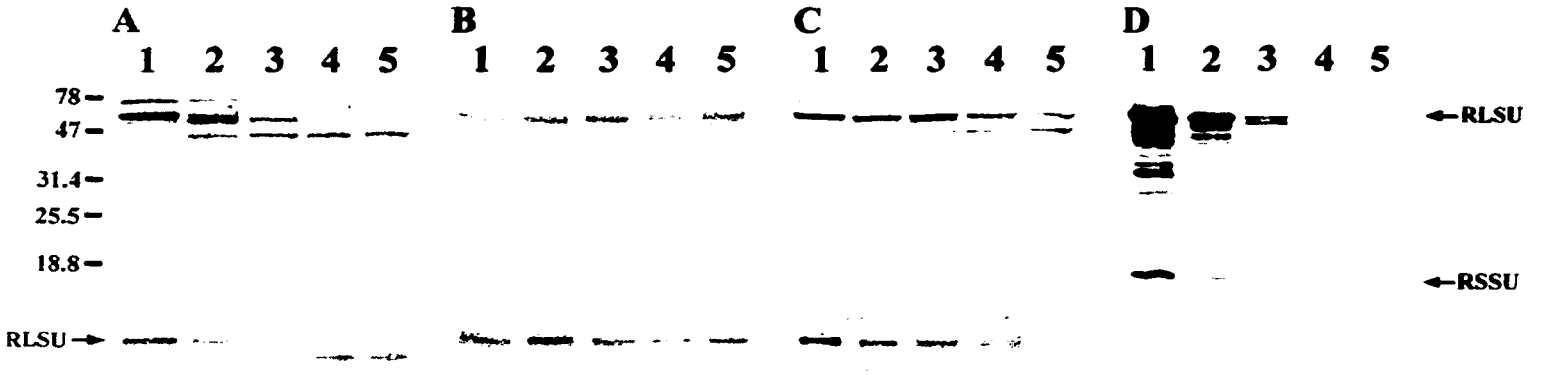
[a]Values are means ± SE for n=3 or 4.

thylakoids. It is clear, therefore, that the immunopurified lipid-protein particles are enriched in free fatty acids relative to thylakoid membranes. This also proved to be true for the full complement of *in vitro*-generated and stromal lipid-protein particles (Table 2.2).

### 2.3.5 Nature of the Association of RLSU with Lipid-Protein Particles

Protease-shaving experiments were conducted in order to determine whether the RLSU associated with immunopurified particles was embedded in the interior of the particle or simply adsorbed to its outside surface. For this purpose, the particles were treated with exogenous proteinase K for up to 120 min, and Western blots of the treated particles were probed with antibody raised against Rubisco holoenzyme. During treatment of *in vitro*-generated lipid-protein particles with proteinase K, the native 55 kD RLSU was progressively converted to a 44 kD protected fragment (Fig. 2.15A, upper panel). This suggests that the 44 kD portion of RLSU is embedded in the interior of lipid-protein particles and thus protected from proteolysis. This is known to be the case, for example, for oleosins associated with oil bodies, which are lipid-protein particles found in oil-bearing seeds (Huang, 1992; Murphy, 1993). RLSU associated with immunopurified stromal lipid-protein particles and with thylakoids proved to be more resistant to protease-shaving. When thylakoids were treated with proteinase K, the 44 kD protected fragment was not detectable initially, and after 120 min only a portion of the native protein had been converted to the protected fragment (Fig. 2.15C, upper panel). When the immunopurified stromal lipid-protein particles were treated with proteinase K, there was no change in the level of native RLSU even after 120 min of treatment, and the 44 kD protected fragment was not detected (Fig. 2.15B, upper panel). When Rubisco holoenzyme was treated with proteinase K, RSSU disappeared within 10 min

Figure 2.15. Western blots of proteinase K-treated lipid-protein particles, thylakoids and Rubisco holoenzyme. (A), *In vitro*-generated lipid-protein particles treated with proteinase K. Lanes were loaded with equal volume (40 μL). Top panel, probed with Rubisco holoenzyme antibody; lower panel, probed with antibody raised against amino acids $A_{430}$-$C_{449}$ of RLSU. Lane 1, 0 min control; lane 2, 1 min proteinase K treatment; lane 3, 10 min proteinase K treatment; lane 4, 60 min proteinase K treatment; lane 5, 120 min proteinase K treatment. (B), Immunoprecipitated stromal lipid-protein particles treated with proteinase K. Panels and lanes are as in (A). (C), Isolated thylakoids treated with proteinase K. Panels and lanes are as in (A). (D), Rubisco holoenzyme (Sigma) treated with proteinase K. Lanes are as in (A). Molecular weight markers (kD) are indicated.


A
B
C
D
1 2 3 4 5
78
47
31.4
25.5
18.8
RLSU
RLSU
RSSU

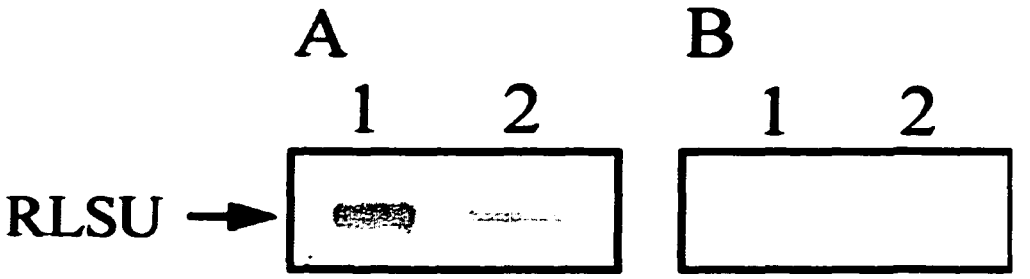
and RLSU within 60 min (Fig. 2.15D). Immunoblots probed with antibodies raised against a peptide from near the C-terminus of RLSU (amino acids $A_{430}$-$C_{449}$) proved to be identical to those probed with antibodies against Rubisco holoenzyme (Figs. 2.15, A, B and C).

The ability of the RLSU-peptide antiserum to react with RLSU purchased from Sigma (isolated from spinach) and from *Phaseolus vulgaris* was confirmed using Western blot analysis. The capacity of the RLSU peptide antibody to bind RLSU is compared to the binding capacity of the pre-immune serum in Figure 2.16. The Western blot shows that there are antibodies in the pre-immune serum that are capable of binding RLSU. This is not unexpected, as Rubisco is the major protein of green tissues, and the rabbits in which the antibodies are raised are fed a vegetarian diet. Therefore, it is expected that there will be Rubisco antibodies present in the serum prior to immunization. However, Figure 2.16 also indicates that the RLSU peptide anti-serum binds to RLSU much more strongly than does the pre-immune serum.

### 2.3.6 Specificity of Association of RLSU with Membranes and Lipid-Protein Particles

The finding that RLSU associated with thylakoids and lipid-protein particles is protected from proteinase K digestion, whereas Rubisco holoenzyme is completely broken down under the same conditions, indicates that the presence of the protected RLSU in these fractions is not attributable to contamination with the holoenzyme. Further evidence supporting this contention was obtained by incubating microsomal membranes and cytosolic lipid-protein particles isolated from etiolated cotyledons of *Phaseolus vulgaris* in stroma that had been rendered free of chloroplast lipid-protein particles by ultrafiltration through a 1,000 kD cut-off filter. The presence of Rubisco holoenzyme in the ultrafiltered stroma was confirmed by

Figure 2.16. **Western blots comparing ability of pre-immune serum antibodies and immune serum antibodies to bind to the large subunit of Rubisco. A, Western blot probed with RLSU-peptide anti-serum, diluted 1:1000. Lane 1, Rubisco holoenzyme purchased from Sigma (1 μg,); lane 2, stroma (2.5 μg total protein). B, Western blot probed with RLSU peptide pre-immune serum, diluted 1:500. Lanes are as in A.**

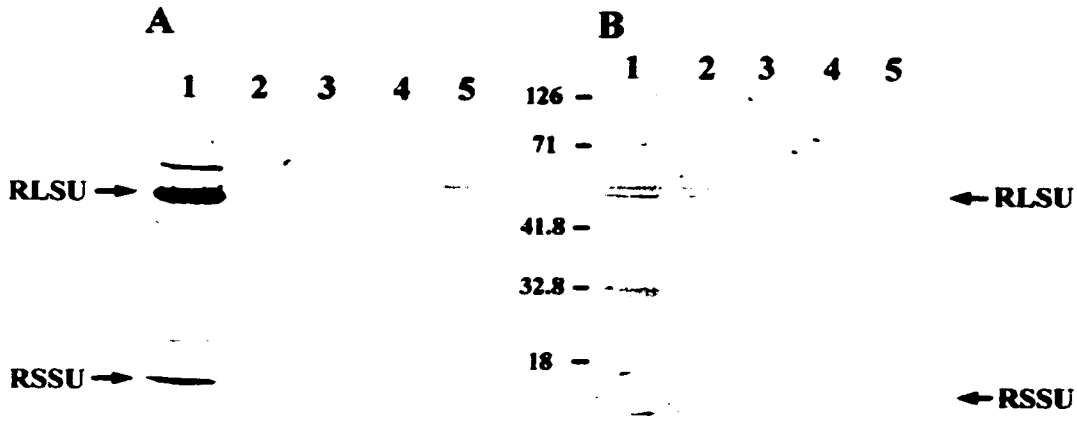

A
B
1
2
1
2
RLSU

Western blot analysis (Fig. 2.17A, lane 1). Microsomal membranes and cytosolic lipid-protein particles were suspended in ultrafiltered stroma at concentrations of 10 mg protein and 0.1 mg protein per mg stromal protein, respectively. These concentration ratios were approximately equivalent to those of thylakoid to stromal protein and stromal lipid-protein particles to stromal protein, respectively, in typical chloroplast preparations. The suspension of microsomal membranes in ultrafiltered stroma was then centrifuged at 2°C for 1 h at 250,000 *g* to re-isolate the microsomal membranes, and cytosolic particles suspended in ultrafiltered stroma were re-isolated by flotation centrifugation. Untreated fractions and fractions re-isolated after treatment were then probed for the presence of Rubisco on Western blots using antibodies against Rubisco holoenzyme. Neither RLSU nor RSSU were detected in Western blots of cytosolic lipid-protein particles before or after treatment (Fig. 2.17A, lanes 2 and 3). Both subunits of Rubisco were detectable on immunoblots of microsomal membranes that had been treated with ultrafiltered stroma (Fig. 2.17A, lane 5), but were completely degraded after 30 min of treatment with exogenous proteinase K (Fig. 2.17B) indicating that they were adsorbed to the surface of the membranes rather than embedded in the interior of the bilayer. Rubisco holoenzyme antibody also cross-reacted with a microsomal membrane protein that is slightly larger than RLSU (~58 kD) and was present prior to incubation with particle-free stroma (Fig. 2.17A, lane 4).

### 2.3.7 Association of Chaperonin 60 with Stromal Lipid-Protein Particles

Western blots of lipid-protein particles isolated from the stroma by flotation centrifugation were also probed with polyclonal cpn60 antibody. Two bands corresponding to the $\alpha$ and $\beta$ subunits of chloroplastic cpn60 (Gatenby and Ellis, 1990; Hemmingson and Ellis,

Figure 2.17. Western blots of microsomal membranes and cytosolic lipid-protein particles isolated from etiolated cotyledons probed with Rubisco holoenzyme antibody. (A), Effect of treatment with particle-free stroma. Lane 1, ultrafiltered particle-free stroma; lane 2, cytosolic lipid-protein particles before treatment; lane 3, cytosolic lipid-protein particles after treatment; lane 4, microsomal membranes before treatment; lane 5, microsomal membranes after treatment. Lanes were loaded with equal protein (1 μg). (B), Effect of treatment of microsomal membranes with particle-free stroma followed by digestion for various periods of time with proteinase K. Lane 1, 0 min control; lane 2, 1 min proteinase K treatment; lane 3, 10 min proteinase K treatment; lane 4, 60 min proteinase K treatment; lane 5, 120 min proteinase K treatment. Lanes were loaded with equal volume (40 μL).


A
1 2 3 4 5
RLSU
RSSU
B
1 2 3 4 5
126
71
41.8
32.8
18
RLSU
RSSU

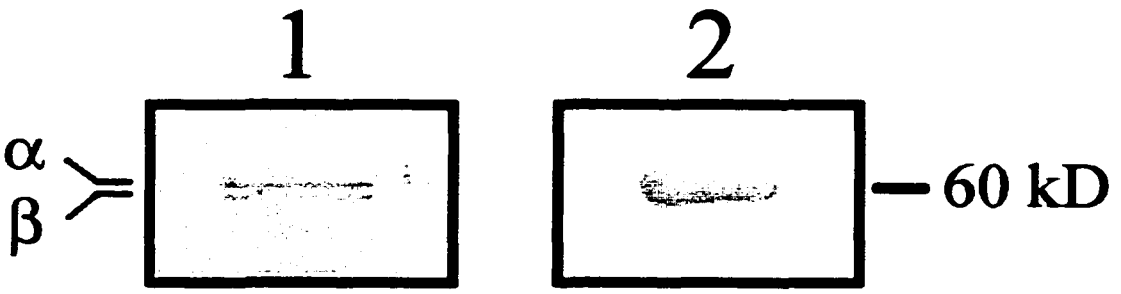
1986) were detectable (Fig. 2.18, lane 1). A band of ~60 kD was also detectable in Western blots of recombinant cpn60 isolated from *E. coli* (Sigma) run in parallel (Fig. 2.18, lane 2).

## 2.4 DISCUSSION

Lipid-protein particles isolated from the stroma of chloroplasts or generated *in vitro* from isolated thylakoids have been reported to contain thylakoid lipids and proteins, and their catabolites (Ghosh et al., 1994). These particles are thought to be formed by blebbing from thylakoids and may serve as a vehicle for removing destabilizing lipid and protein catabolites from the thylakoid bilayer (Ghosh et al., 1994). In the present study, it is reported that a subpopulation of both stromal lipid-protein particles and those generated *in vitro* from isolated thylakoids contain the large subunit of Rubisco, but no detectable corresponding small subunit. These observations can be interpreted as indicating that RLSU is released from thylakoids in association with lipid-protein particles that are blebbed from the membrane surface. Moreover, the release is largely heat-denaturable and facilitated by light and ATP, suggesting that it is physiologically regulated.

Several lines of evidence support the contention that Rubisco large subunit in the lipid-protein particle fraction is associated with lipid and not present simply as a free polypeptide or as Rubisco holoenzyme. First, Western blot analysis indicated that *in vitro*-generated lipid-protein particles are enriched in RLSU relative to thylakoids and that RSSU is not detectable in either fraction. As well, a subpopulation of lipid-protein particles isolated from stroma by flotation also contains RLSU but no detectable RSSU. Second, when lipid-protein particles were fractionated by Sephacryl size-exclusion chromatography, RLSU eluted in fractions

Figure 2.18. Western blots of lipid-protein particles isolated from the stroma by flotation centrifugation and purified cpn60 probed with polyclonal cpn60 antibody. Lane 1, lipid-protein particles isolated from the stroma by flotation centrifugation; lane 2, recombinant cpn60 isolated from *E. coli* (purchased from Sigma). The $\alpha$ and $\beta$ subunits of chloroplastic cpn60 are indicated for lane 1.


1
2
α
β
60 kD

corresponding to a molecular weight of ~550 kD, whereas free RLSU polypeptide eluted in fractions corresponding to its expected molecular mass of ~55 kD. This elution pattern could be interpreted as indicating that the RLSU is incorporated into Rubisco holoenzyme rather than associated with particles. However, Western blots of these RLSU-containing fractions confirmed the absence of RSSU, and therefore preclude this possibility. Inasmuch as Sephacryl chromatography resolves molecules in accordance with Stokes' radius, the RLSU-containing particles, which are ~100 nm in diameter, should elute ahead of Rubisco holoenzyme. However, the particles contain lipid which may alter their elution rate relative to pure proteins of a similar size, and Rubisco holoenzyme approximates a cube with rounded edges, which equates to a Stokes' radius larger than its actual size (Hartman and Harpel, 1994; Andrews, 1970). As well, molecular weight markers of 550 kD and 670 kD were not well resolved on the column indicating that separation in this size range is beyond the effective resolution capability of Sephacryl S-300 HR. Third, immunopurification of RLSU from the unfractionated population of both *in vitro*-generated lipid-protein particles and those isolated from the stroma by flotation centrifugation confirmed that it is associated with other proteins and lipid, and that the fatty acids of the lipid are also found in the thylakoid membrane. Finally, RLSU associated with thylakoids and lipid-protein particles proved to be resistant to digestion by proteinase K, whereas that associated with Rubisco holoenzyme was completely degraded. Indeed, both subunits of Rubisco were completely digested when the holoenzyme was treated with proteinase K. Small amounts of Rubisco became associated with microsomal membranes that were resuspended in stroma, but both subunits were completely degraded when the membranes were subsequently treated with proteinase K indicating that the holoenzyme had simply adsorbed to the membrane

surface. Neither subunit of Rubisco was detectable in cytosolic lipid-protein particles that had been treated with stroma.

These observations are consistent with the contention that protease-protected RLSU is intrinsically associated with thylakoids *in situ* and can be released into the stroma by blebbing of lipid-protein particles from the membrane surface. The orientation of RLSU within thylakoid membranes and lipid-protein particles was assessed by Western blotting using antibodies raised against the C-terminal end of RLSU. Treatment of thylakoids and of *in vitro*-generated lipid-protein particles with proteinase K converts the 55 kD native RLSU to a 44 kD protease-protected fragment. This could arise if the protease clipped ~11 kD from one end of the polypeptide or smaller portions from both termini. The C terminus-specific antibodies were raised against a 20-amino acid peptide corresponding to residues $A_{430}$ to $C_{449}$, which would be within the first 5 kD C-terminal segment of the polypeptide. Neither of the RLSU termini are likely to be embedded in the hydrophobic interior of the thylakoids or particles inasmuch as they are both polar in nature. Thus the finding that the Western blots of proteinase K-treated thylakoids and lipid-protein particles probed with the C-terminus-specific antibodies were identical to those probed with holoenzyme antibodies suggests that the protease clips small amounts from both the N-terminus and the C-terminus. Further, inasmuch as the particles have only one surface unlike thylakoids which have both a stromal and a lumenal surface, these observations suggest that the RLSU termini are exposed on the same surface of thylakoids, most likely the stromal surface, inasmuch as the particles appear to be released into the stroma. The proteinase K shaving experiments also indicate that the termini of RLSU in the stromal particles are less accessible to the protease and hence more cryptic than their counterparts in thylakoids and *in vitro*-generated lipid-protein particles.

Several previous findings are consistent with the view that Rubisco large subunit may have a transient association with thylakoids. For example, the coat protein of TMV-PV230, which is known to associate with thylakoids, has significant homology with RLSU (Reinero and Beachy, 1986). Large subunit mRNA (*rbcL*) also has sequence motifs in common with the mRNAs of some chloroplastically encoded thylakoid proteins, including the β subunit of ATP synthase (*atpB*), the core complex of photosystem I (*psaA*) and the D1 protein of photosystem II (*psbA*) (Kim et al., 1993). In addition, RLSU, like chloroplastically encoded thylakoid proteins, appears to be translated by thylakoid-bound polysomes associated with stromal lamellae and the outer margins of granal stacks (Yamamoto et al., 1981; Bhaya and Jagendorf, 1984; Hattori and Margulies, 1986; Klein et al., 1988). It is believed that thylakoid-bound polysomes are capable of synthesizing both thylakoid and stromal proteins, but there is uncertainty as to how much RLSU is formed on thylakoid-bound ribosomes, as distinct from soluble polysomes (Jagendorf and Micheals, 1990). However, it seems clear that at least a portion of Rubisco large subunit is synthesized on thylakoid-bound polysomes and may associate with thylakoids prior to incorporation into Rubisco holoenzyme (Bhaya and Jagendorf, 1984; Hattori and Margulies, 1986; Makino and Osmond, 1991). There is also evidence that thylakoid-bound polysomes engage in co-translational insertion into thylakoids (Michaels and Margulies, 1975; Kim et al., 1991). Moreover, the large subunit of Rubisco is a relatively insoluble protein (Gatenby, 1984) and is synthesized as a precursor polypeptide from which an N-terminal fragment of about 14 amino acids is post-translationally removed prior to its assembly into Rubisco holoenzyme (Langridge, 1981; Amiri et al., 1984).

The finding in the present study that RLSU is discernible in Western blots of washed thylakoids and appears to be released by blebbing of lipid-protein particles is also consistent with

the concept that it becomes associated with thylakoid membranes prior to being assembled into Rubisco holoenzyme. The precise nature of this association is not clear, but the fact that the release of RLSU by blebbing is largely heat-denaturable and facilitated by light and ATP suggests that the polypeptide has not simply become adsorbed to the thylakoid surface. This contention is supported by the fact that thylakoid RLSU is resistant to protease shaving, and is not removed from thylakoids by washing with 0.1 M $Na_2CO_3$ (pH 11.0) or treatment with 0.5% Triton X-100.

It seems possible that light-facilitated blebbing from thylakoids of lipid-protein particles bearing RLSU is coordinated *in situ* with the assembly of Rubisco holoenzyme. This contention is supported by the finding that cpn60 is also associated with lipid-protein particles isolated from the stroma by flotation centrifugation. The exact nature of the association between cpn60 and the particles remains to be elucidated; in particular, it has not yet been determined whether cpn60 is directly associated with RLSU in the particles. Nevertheless, the presence of cpn60 suggests that these stromal particles may be involved in Rubisco holoenzyme assembly. Indeed, the lipid-protein particles may provide the hydrophobic environment needed for formation of the soluble eight large-subunit core of Rubisco from insoluble large subunits. However, the possibility that large subunit found embedded in thylakoids and lipid-protein particles represents improperly folded RLSU, which has dissociated from cpn60 during Rubisco assembly, aggregated and is destined for degradation rather than assembly into holoenzyme, is not precluded (Roy et al., 1982). This seems unlikely, though, given that such aggregates would more likely adsorb to the hydrophilic surfaces of membranes and stromal particles than penetrate into their hydrophobic interiors and, as such, not be resistant to protease-shaving.

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# 3. Co-Association of Cytochrome *f* Catabolites and PAP (Plastid-lipid Associated Protein) with Chloroplast Lipid Particles[1]

## 3.1 INTRODUCTION

Plastoglobuli are lipid bodies found in all types of plastids. They have been extensively described, yet their exact structure and chemical composition are not known with certainty (Lichtenthaler, 1968; Hansmann and Sitte, 1982; Steinmüller and Tevini, 1985a; Pozueta-Romero et al., 1997). It is not clear, for example, whether plastoglobuli are circumscribed by a polar lipid monolayer analogous to the phospholipid monolayer that surrounds cytosolic oil bodies from seeds and other organs (Murphy, 1993; Huang, 1996). This uncertainty stems from conflicting reports about the presence of galactolipids in plastoglobuli and whether it is possible to visualize a half-unit membrane by electron microscopy (Greenwood et al., 1963; Simpson and Lee, 1976; Hansmann and Sitte, 1982; Steinmüller and Tevini, 1985a). A recent study of tapetal cell elaioplasts provides more definitive evidence for a monolayer of galactolipids surrounding the neutral lipid core of plastoglobuli, and indicates that these lipid bodies originate from thylakoids (Hernández-Pinzón et al., 1999). This evidence is in agreement with a model for plastid lipid body organization proposed by Knoth et al. (1986).

There are also conflicting reports regarding the presence of proteins in plastoglobuli. Steinmüller and Tevini (1985a) have suggested that protein associated with isolated plastoglobuli is artifactual, whereas others have argued that numerous proteins are native constituents of plastoglobuli (Bailey and Whyborn, 1963; Hansmann and Sitte, 1982; Kessler

[1] The work presented in this chapter was submitted for publication in *Plant Physiology* on December 23, 1999 with co-authors Donny D. Licatalosi and John E. Thompson.

et al., 1999; Hernández-Pinzón et al., 1999). Some proteins associated with plastoglobuli appear to be members of a family of proteins characterized as lipid-associated proteins. These include plastid-lipid associated protein (PAP), fibrillin, plastoglobulin 1, carotenoid-associated protein, carotene globule protein and the 32- and 34-kDa chloroplast drought-induced stress proteins (Deruère et al., 1994; Katz et al., 1995; Vishnevetsky et al., 1996; Pozueta-Romero et al., 1997; Kessler et al., 1999; Eymery and Rey, 1999; Hernández-Pinzón et al., 1999). These proteins range in size from 30-38 kD and are thought to be involved in maintaining the structural stability of plastid lipid bodies (Ting et al., 1998). In light of accumulating evidence for their existence, it seems likely that these proteins are not only genuine components of plastoglobuli, but also serve as markers for plastid lipid bodies.

The functional role of plastoglobuli has not been conclusively established (Lichtenthaler, 1968; Tuquet and Newman, 1980). However, it is assumed based on a reduction in their size and abundance during thylakoid biogenesis and their accumulation and increase in size during thylakoid degradation that they store thylakoid components, especially those liberated during dissolution of the thylakoid membrane (Sprey and Lichtenthaler, 1966; Lichtenthaler, 1968; Lichtenthaler and Weinert, 1970). Indeed, plastoglobuli isolated from senescing leaves are enriched in thylakoid lipid metabolites, particularly free fatty acids (Steinmüller and Tevini, 1985b). Plastoglobuli may also function as a depot for surplus lipids in general (Greenwood et al., 1963; Thomson and Platt, 1973). More recent evidence suggests that plastoglobuli of senescing chloroplasts are exuded through the chloroplast envelope into the cytoplasm and subsequently degraded (Guiamét et al., 1999).

Differences in lipid composition between plastoglobuli from chloroplasts and chromoplasts, and even among chloroplastic plastoglobuli, have been interpreted as reflecting subpopulations of plastoglobuli (Simpson and Lee, 1976). In an earlier study, Bailey and

Whyborn (1963) characterized two classes of lipid particles in chloroplasts of *Beta vulgaris* leaves that were distinguishable on the basis of differences in density. More recently, plastoglobuli of differing densities were isolated from chloroplasts of *Pisum sativum* leaves using a sucrose gradient (Kessler et al., 1999). It has also been proposed that differences in the electron density of plastoglobuli in the chloroplasts of some species, for example, *Capsicum annuum*, reflect differences in chemical composition (Simpson and Lee, 1976).

Another class of lipid bodies, termed lipid-protein particles, has been isolated from the stroma of chloroplasts from mature *Phaseolus vulgaris* leaves (Ghosh et al., 1994; Smith et al., 1997). These particles contain thylakoid proteins and their metabolites as well as other chloroplast proteins, and are also enriched in thylakoid lipid catabolites, in particular free fatty acids (Ghosh et al., 1994; Smith et al., 1997). It has been proposed that these particles are formed from thylakoids and play an integral role in normal thylakoid turnover, allowing removal of thylakoid protein and lipid catabolites that would otherwise destabilize the bilayer (Ghosh et al., 1994; Thompson et al., 1998).

In the present study, plastoglobuli and higher-density stromal lipid-protein particles have been isolated from chloroplasts of *Phaseolus vulgaris* leaves. They both contain the plastoglobuli-specific protein, PAP, indicating that the stromal lipid-protein particles are plastoglobuli-like particles, and they also contain catabolites of the thylakoid protein, cytochrome *f*. The results suggest that plastoglobuli and plastoglobuli-like lipid particles may be involved in thylakoid turnover, allowing removal of protein catabolites from the thylakoid membrane that are destined for degradation.

## 3.2 MATERIALS AND METHODS

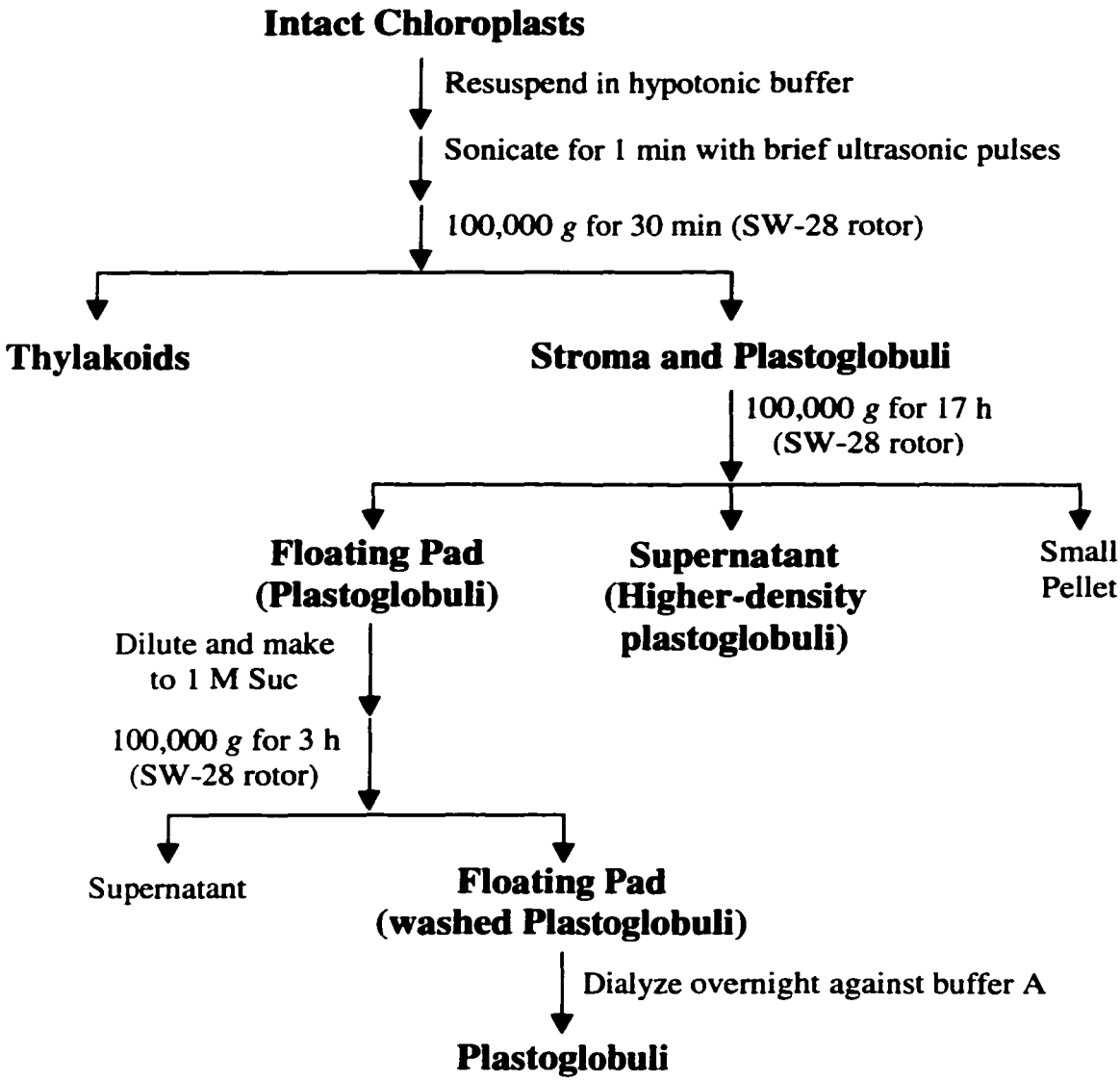
### 3.2.1 Plant Material and Isolation of Chloroplasts

Yellow wax beans (*Phaseolus vulgaris* L. cv Kinghorn Wax) were grown in flats of Pro-mix BX (Premier Brands) under greenhouse conditions with a supplementary 16-h photoperiod of fluorescent light (Chapter 2). Intact chloroplasts were isolated from the primary leaves of 14-d-old seedlings as described previously (Chapter 2).

### 3.2.2 Isolation of Plastoglobuli and Stromal Lipid-Protein Particles

Plastoglobuli were isolated as illustrated in Figure 3.1 using an established protocol (Bailey and Whyborn, 1963). Intact chloroplasts were suspended in hypotonic lysis buffer containing 10 mM Epps-KOH (pH 7.8), 10 mM $MgCl_2$ and 10 mM $NaHCO_3$, and the plastoglobuli were released from thylakoids by short pulses of sonication while being held on ice, for 1 min using a Model 450 Sonifier (Branson Ultrasonics Corp., Danbury, CT) operating at 20 kHz. The sonicated suspension was centrifuged at 100,000*g* for 30 min in an SW-28 rotor (Beckman) to pellet the thylakoid membranes, and the resulting supernatant containing the stroma and plastoglobuli was centrifuged again in an SW-28 rotor at 100,000*g* for 17 h. A floating pad of plastoglobuli formed during this centrifugation. The supernatant, which contained plastoglobuli of higher density that did not float, was collected (from top to bottom) as four sequential fractions of equal volume termed F1, F2, F3 and F4, respectively, and retained for analysis. The floated plastoglobuli were washed by dilution in buffer A (50 mM Epps-KOH, pH 7.8, 10 mM $MgCl_2$, 10 mM $NaHCO_3$, 250 mM D-sorbitol and 1% (v/v) glycerol), and sucrose was added to a final concentration of 1 M. The suspension was then

Figure 3.1. **Flow-chart illustrating the isolation of floated plastoglobuli and higher-density plastoglobuli from sonicated chloroplasts.**


Intact Chloroplasts
Resuspend in hypotonic buffer
Sonicate for 1 min with brief ultrasonic pulses
100,000 g for 30 min (SW-28 rotor)
Thylakoids
Stroma and Plastoglobuli
100,000 g for 17 h
(SW-28 rotor)
Floating Pad
(Plastoglobuli)
Supernatant
(Higher-density
plastoglobuli)
Small
Pellet
Dilute and make
to 1 M Suc
100,000 g for 3 h
(SW-28 rotor)
Supernatant
Floating Pad
(washed Plastoglobuli)
Dialyze overnight against buffer A
Plastoglobuli

centrifuged at 100,000*g* for 3 h in an SW-28 rotor. The resulting floating pad of washed plastoglobuli was collected and dialyzed against buffer A.

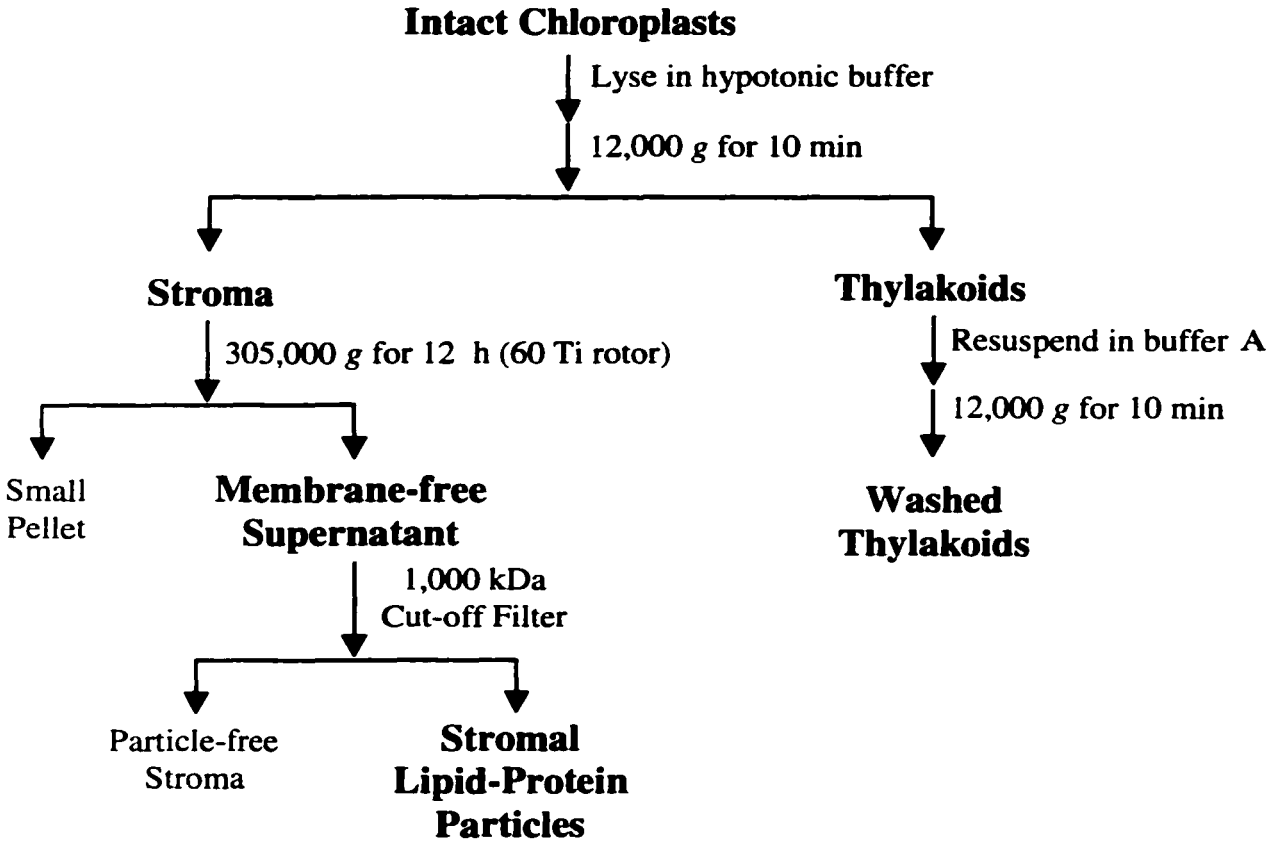
Higher density stromal lipid-protein particles were isolated as previously described (Ghosh et al., 1994) from chloroplasts that had not been sonicated (Fig. 3.2). Intact chloroplasts were suspended in hypotonic lysis buffer and incubated for 30 min on ice in the dark. Lysis was stopped by the addition of an equal volume of double-strength buffer A. The suspension was centrifuged at 12,000*g* for 10 min to yield pelleted thylakoids and a supernatant containing the stroma. The stroma was centrifuged at 305,000*g* for 12 h in a 60-Ti rotor (Beckman) to remove any residual membranes. The stromal lipid-protein particles remained in suspension and were concentrated by passing the supernatant through a 1,000-kD cut-off filter (Fig. 3.2).

### 3.2.3 Antisera

Dr. Shimon Gepstein (Technion-Israel Institute of Technology, Israel) kindly provided antibodies raised in rabbit against mature, full-length, SDS-PAGE-purified cytochrome *f* from spinach thylakoids. Antibodies against PAP from *Capsicum annuum* were a generous gift from Dr. Rudolphe Schantz (Centre National de la Researche Scientifique, France), and were raised in rabbit against a PAP-glutathione S-transferase fusion protein expressed in *E. coli* (Pozueta-Romero et al., 1997).

Antibodies specific for both termini of cytochrome *f* were raised in rabbits against synthetic peptides conjugated to the carrier protein, Keyhole Limpet Hemocyanin (KLH). The N-terminal-specific antiserum was generated against a 21-amino acid peptide ($H_2N$-

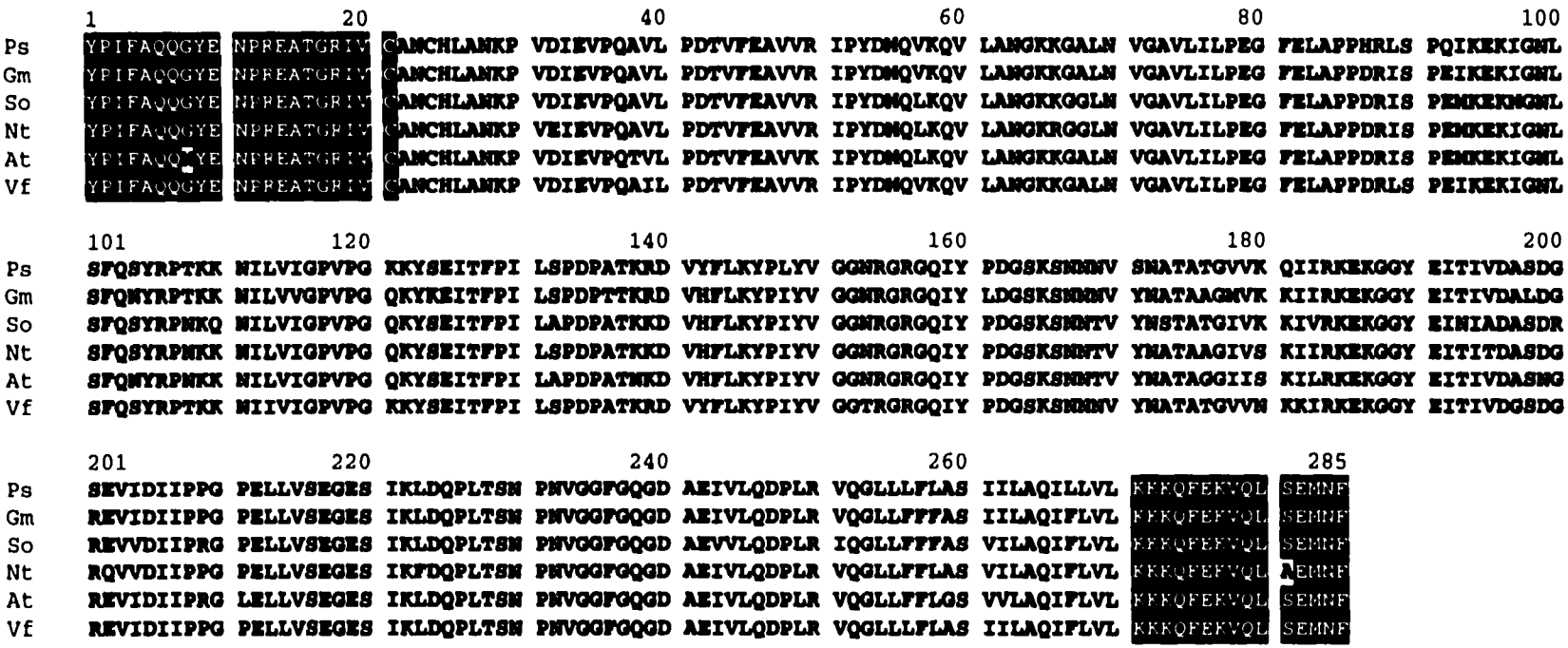
Figure 3.2. **Flow-chart illustrating the isolation of lipid-protein particles from the stroma of non-sonicated chloroplasts.**


Intact Chloroplasts
Lyse in hypotonic buffer
12,000 g for 10 min
Stroma
Thylakoids
305,000 g for 12 h (60 Ti rotor)
Resuspend in buffer A
12,000 g for 10 min
Small Pellet
Membrane-free Supernatant
Washed Thylakoids
1,000 kDa Cut-off Filter
Particle-free Stroma
Stromal Lipid-Protein Particles

YPIFAQQGYENPREATGRIVC-COOH) corresponding to the N-terminus of mature cytochrome *f*. This peptide was selected because the termini of proteins are often particularly immunogenic (Drenckhahn et al., 1993), and cytochrome *f* is highly conserved among higher plants in this region (Fig. 3.3). The region consists mainly of hydrophilic amino acids (hydrophilicity equates positively with antigenicity), and the cysteine residue at the C-terminus of the peptide allowed for conjugation to the carrier protein. The C-terminal-specific antiserum was generated against a 16-amino acid peptide ($H_2N$-CKKKQFEKVQLSEMNF-COOH) corresponding to the highly conserved 15 C-terminal residues of mature cytochrome *f* (Fig. 3.3) that protrude into the stroma, plus an additional cysteine residue added to the amino-terminus of the peptide to enable coupling to the carrier protein. The majority of residues in this peptide are also hydrophilic, and the peptide is likely to be antigenic as it corresponds to the extreme C-terminus of the protein (Drenckhahn et al., 1993). The peptides were synthesized in the laboratory of Dr. G. Lajoie (Dept. of Chemistry, University of Waterloo).

Conjugation of peptides to carrier proteins was necessary to facilitate an immune response in rabbits, and was accomplished through the terminal cysteine residues of the peptides using *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (MBS) (Drenckhahn et al., 1993; Collawn and Patterson, 1999). MBS is a heterobifunctional agent that cross-links terminal thiol groups (provided by the Cys residue of the peptide) with Lys side chains in the carrier protein. To accomplish cross-linking, KLH was dissolved in 0.1 M sodium phosphate buffer (0.1 M $Na_2HPO_4$, 0.1 M $NaH_2PO_4$, pH 6.8) at a concentration of 1 mg/mL in a small glass test tube. One-fifth volume of freshly prepared dimethylformamide (DMF)-MBS coupling solution (containing 10 mg MBS per mL DMF) was added to the dissolved KLH and gently stirred using a small magnetic stir bar for 30 min at room temperature. The mixture was then

Figure 3.3. Aligned amino acid sequences of mature cytochrome *f* proteins from six higher plants highlighting the regions corresponding to the synthetic peptides that were used as antigens to generate polyclonal antibodies. Ps, *Pisum sativum*; Gm, *Glycine max*; So, *Spinacia oleracea*; Nt, *Nicotiana tabacum*; At, *Arabidopsis thaliana*; Vf, *Vicia faba*. The numbers correspond to amino acid residues, beginning with the N-terminus. The sequences were aligned using MultAlin version 5.3.3 (Corpet, 1988), a multiple sequence alignment program available on the world wide web (W3.toulouse.inra.fr).


1 20 40 60 80 100
Ps YPIFAQQGYE NPREATGRIV GAMCHLAMKP VDIEVPQAVL PDTVFEAVVR IPYDMQVKQV LAMGKKGALM VGAVLILPEG FELAPPHRLS PQIKEKIGML
Gm YPIFAQQGYE NPREATGRIV GAMCHLAMKP VDIEVPQAVL PDTVFEAVVR IPYDMQVKQV LAMGKKGALM VGAVLILPEG FELAPPDRIS PEIKEKIGML
So YPIFAQQGYE NPREATGRIV GAMCHLAMKP VDIEVPQAVL PDTVFEAVVR IPYDMQLKQV LAMGKKGGLM VGAVLILPEG FELAPPDRIS PEMKEKMGML
Nt YPIFAQQGYE NPREATGRIV GAMCHLAMKP VEIEVPQAVL PDTVFEAVVR IPYDMQLKQV LAMGKRGGLM VGAVLILPEG FELAPPDRIS PEMKEKIGML
At YPIFAQQGYE NPREATGRIV GAMCHLAMKP VDIEVPQTVL PDTVFEAVVK IPYDMQLKQV LAMGKKGALM VGAVLILPEG FELAPPDRIS PEMKEKIGML
Vf YPIFAQQGYE NPREATGRIV GAMCHLAMKP VDIEVPQAIL PDTVFEAVVR IPYDMQVKQV LAMGKKGALM VGAVLILPEG FELAPPDRLS PEIKEKIGML
101 120 140 160 180 200
Ps SFQSYRPTKK MILVIGPVPG KKYSEITFPI LSPDPATKRD VYFLKYPLYV GGMRGRGQIY PDGSKSMMMV SMATATGVVK QIIRKEKGGY EITIVDASDG
Gm SFQMYRPTKK MILVVGPVPG QKYKEITFPI LSPDPTTKRD VHFLKYPIYV GGMRGRGQIY LDGSKSMMMV YMATAAGMVK KIIRKEKGGY EITIVDALDG
So SFQSYRPMKQ MILVIGPVPG QKYSEITFPI LAPDPATKKD VHFLKYPIYV GGMRGRGQIY PDGSKSMMTV YMSTATGIVK KIVRKEKGGY EIMIADASDR
Nt SFQSYRPMKK MILVIGPVPG QKYSEITFPI LSPDPATKKD VHFLKYPIYV GGMRGRGQIY PDGSKSMMTV YMATAAGIVS KIIRKEKGGY EITITDASDG
At SFQMYRPMKK MILVIGPVPG QKYSEITFPI LAPDPATMKD VHFLKYPIYV GGMRGRGQIY PDGSKSMMTV YMATAGGIIS KILRKEKGGY EITIVDASMG
Vf SFQSYRPTKK MIIVIGPVPG KKYSEITFPI LSPDPATKRD VYFLKYPIYV GGTRGRGQIY PDGSKSMMMV YMATATGVVM KKIRKEKGGY EITIVDGSDG
201 220 240 260 285
Ps SEVIDIIPPG PELLVSEGES IKLDQPLTSN PMVGGFGQGD AEIVLQDPLR VQGLLLFLAS IILAQILLVL KFKQFEKVQL SEMNF
Gm REVIDIIPPG PELLVSEGES IKLDQPLTSN PMVGGFGQGD AEIVLQDPLR VQGLLFFFAS IILAQIFLVL KFKQFEKVQL SEMNF
So REVVDIIPRG PELLVSEGES IKLDQPLTSN PMVGGFGQGD AEVVLQDPLR IQGLLFFFAS VILAQIFLVL KFKQFEKVQL SEMNF
Nt RQVVDIIPPG PELLVSEGES IKFDQPLTSN PMVGGFGQGD AEIVLQDPLR VQGLLFFLAS VILAQIFLVL KKKQFEKVQL AEMNF
At REVIDIIPRG LELLVSEGES IKLDQPLTSN PMVGGFGQGD AEIVLQDPLR VQGLLFFLGS VVLAQIFLVL KFKQFEKVQL SEMNF
Vf REVIDIIPPG PELLVSEGES IKLDQPLTSN PMVGGFGQGD AEIVLQDPLR VQGLLLFLAS IILAQIFLVL KKKQFEKVQL SEMNF

loaded onto a Pharmacia C-10 column (1.0 cm i.d. x 40 cm) containing Sephadex G-25 equilibrated with 0.1 M sodium phosphate buffer, to separate activated KLH (KLH-MBS complex) from free MBS. Fractions of 15 drops (<1 mL) were collected, and the relative protein content was measured by reading the $A_{280}$ of each. The fractions comprising the first absorbance peak contained the KLH-MBS complex (activated KLH) and were pooled (Collawn and Patterson, 1999). The pH of the pooled fractions was adjusted to 7.4 and 1 mg of peptide was added per mg of KLH. The tube was sealed under $N_2$, and stirred gently using a small magnetic stir bar for 3-4 h at room temperature to allow for conjugation of peptide to the activated KLH-MBS complex. The resulting peptide-KLH conjugate was dialyzed overnight at 4°C against 4 L of phosphate-buffered saline (PBS). The following day, dialysis was continued for 4 more hours against fresh PBS. Polyclonal antisera were prepared in rabbits housed in the Department of Biology's animal care facility according to the University of Waterloo Animal Care Committee's Standard Operating Procedure. For primary immunization, an emulsion of 500 µL conjugated peptide solution (at a concentration of 2 mg total protein per mL) in an equal volume of Freund's complete adjuvant was prepared. Similar emulsions were prepared using Freund's incomplete adjuvant for subsequent booster injections.

#### *3.2.3.1 Titer Determination of Terminus-Specific Polyclonal Antibodies*

The reactivity of the two terminus-specific antibodies was tested using the dot-blot method of Drenckhahn et al. (1993) before they were used for Western blot analysis. Specifically, 2 µL aliquots of the appropriate pure peptide or full-length cytochrome *f* isolated from spinach (purchased from Sigma) each containing 1 µg of total protein were spotted onto small pieces of nitrocellulose. The nitrocellulose was then blocked using PBS containing 5% (w/v) powdered

milk for 1 h and rinsed three times for 10 min each, once with PBS, once with PBS containing 1% (w/v) powdered milk, and again with PBS. The pieces of nitrocellulose were then incubated separately with antiserum or pre-immune serum diluted in PBS containing 1% (w/v) powdered milk 1:500, 1:1000, 1:2000 (or 1:2500), 1:5000 and up to 1:10000, for 1 h at room temperature with constant agitation. The nitrocellulose was then washed, incubated with secondary antibody and developed as a typical Western blot as described in Chapter 2 or section 3.2.4 (see below).

### 3.2.4 SDS-PAGE and Western Blotting

Proteins were fractionated by SDS-PAGE in 12% gels (Laemmli, 1970 and Chapter 2) and either stained with silver (Wray et al., 1981) or transferred to PVDF membranes using the semi-dry transfer method (Bio-Rad semi-dry transfer cell) and the same electrophoretic transfer buffer used for wet-transfer (see Chapter 2). Following transfer, the blots were blocked by treatment for 30 s with 1 µg/mL polyvinyl alcohol (Miranda et al., 1993) and subsequent incubation for 30 min in phosphate-buffered saline containing 0.05% (v/v) Tween-20 (PBS-T) and 5% (w/v) powdered milk. The blots were then probed with primary antibody, appropriately diluted in PBS-T containing 1% (w/v) powdered milk, for 1 h at room temperature. Following incubation in primary antibody, the blots were washed three times for ten minutes each in PBS-T, PBS-T containing 1% (w/v) powdered milk, and again in PBS-T. Antigens were visualized by incubating the blots in secondary antibody coupled to horseradish peroxidase (DAKO Corp., Carpenteria, CA) diluted 1:10000 in PBS-T containing 1% (w/v) powdered milk for 1 h and using a chemiluminescence detection system (Boehringer Mannheim Chemiluminescence Blotting Substrate [POD]). Specifically, following incubation in secondary antibody, the blots were washed with PBS-T for 10 min, followed by two more

10-min washes, one in PBS-T plus 1% (w/v) powdered milk, and again in PBS-T. The blots were then drained of excess buffer and placed face-up in a fresh disposable weighing tray. One milliliter of freshly prepared, premixed detection reagent (solution A and solution B mixed in a ratio of 100:1) was then applied to the surface of each blot, and allowed to incubate for 1 min at room temperature. The blots were drained of excess reagent and placed between two pieces of clear acetate. Working in a dark room and using a light-tight autoradiography cassette, the blots were exposed to X-ray film (Kodak XL1-Blue) for as little as 10 sec and as long as 2 min, depending on the strength of the chemiluminescent signal. The film was then developed using standard film-developing solutions and techniques.

Some blots were stripped and re-probed with another primary antibody. This was achieved by soaking the PVDF membrane in PBS containing 2% (w/v) SDS and 100 mM β-mercaptoethanol for 15 min at 55°C. After rinsing with a large volume of water, the blots were washed for 15 min with PBS, blocked using 5% (w/v) powdered milk in PBS and re-probed with new primary antibody.

### 3.2.5 Expression of a Recombinant Fragment of Cytochrome *f* in *E. coli*

A 741 bp fragment of the cytochrome *f* gene (*petA*) from *Vicia faba* (Ko and Straus, 1987) corresponding to the globular N-terminal domain of the mature protein was expressed in *E. coli*. The gene fragment was amplified by PCR using an upstream primer (5′-CCC ATT TCC ATG GCA TAT CCT ATT TTT GCC C-3′) containing an *Nco*I restriction site extension (underlined), a downstream primer (5′-G GAC ACG AAG CTT ATC TTG AAG CAC TAT TTC-3′) containing a *Hind*III restriction site extension (underlined), and the full-length *petA* gene from *V. faba* as a template. The positions of the primers are shown on the amino acid and

corresponding nucleotide sequence of *V. faba* cytochrome *f* in Figure 3.4. Additional nucleotides were incorporated into the 5′ end of each primer to facilitate efficient formation of cohesive ends on the PCR product by the restriction enzymes *Nco*I and *Hind*III for sub-cloning. The upstream primer allowed incorporation of an ATG start codon into the truncated *petA* gene by reason of its *Nco*I restriction site extension (5′-C^CATGG). This was necessary since the 5′ end of the truncated gene corresponds to the N-terminus of the mature protein formed by cleavage of the 35 amino acid thylakoid-targeting sequence of apocytochrome *f* (Gray, 1992) and therefore lacks a transcription initiation codon. Using a programmable thermal cycler (Perkin Elmer, GeneAmp PCR System 2400), amplification was performed for 35 cycles, which consisted of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 2 min, with a final extension at 72°C for 15 min. The amplified PCR product was digested with *NcoI*/*Hind*III and cloned into the multiple-cloning site of the expression vector, p*Trc* 99A (Fig. 3.5, Amersham-Pharmacia; kindly provided by Dr. B. Moffatt, Department of Biology, University of Waterloo), which had been digested with the same restriction enzymes, creating p*Trc* 99A-*petA*1. *E. coli* DH5-α cells made competent using the RbCl/$CaCl_2$ method (Kushner, 1978) and transformed with p*Trc* 99A-*petA*1 were grown to $A_{600}$ = 0.6, and expression of the truncated *petA* gene was induced by treatment with 1 mM isopropyl-1-thio-β-D-galactopyranoside for 3 h at 30°C. Cell lysate containing the cytochrome *f* recombinant protein was isolated as described by Sambrook et al. (1989). Specifically, cells were harvested by centrifugation at 3500 *g* for 15 min and lysed by resuspension in bacterial lysis buffer (50 mM Tris (pH 8.0), 100 mM NaCl, and 1 mM EDTA) containing 130 μg/mL PMSF and 500 μg/mL lysozyme, and incubation at room temperature

Figure 3.4. Nucleotide and corresponding amino acid sequence of the full-length apocytochrome *f* (*petA*) clone from *Vicia faba* (Ko and Straus, 1987). The locations of the primers used for PCR amplification of the portion of the clone corresponding to the N-terminal hydrophilic globular domain of the protein are indicated. Restriction endonuclease recognition site linkers (*Nco*I and *Hind*III) were incorporated into the 5′ ends of both primers to facilitate sub-cloning of the resulting *petA* gene fragment into the p*Trc* 99A expression vector.

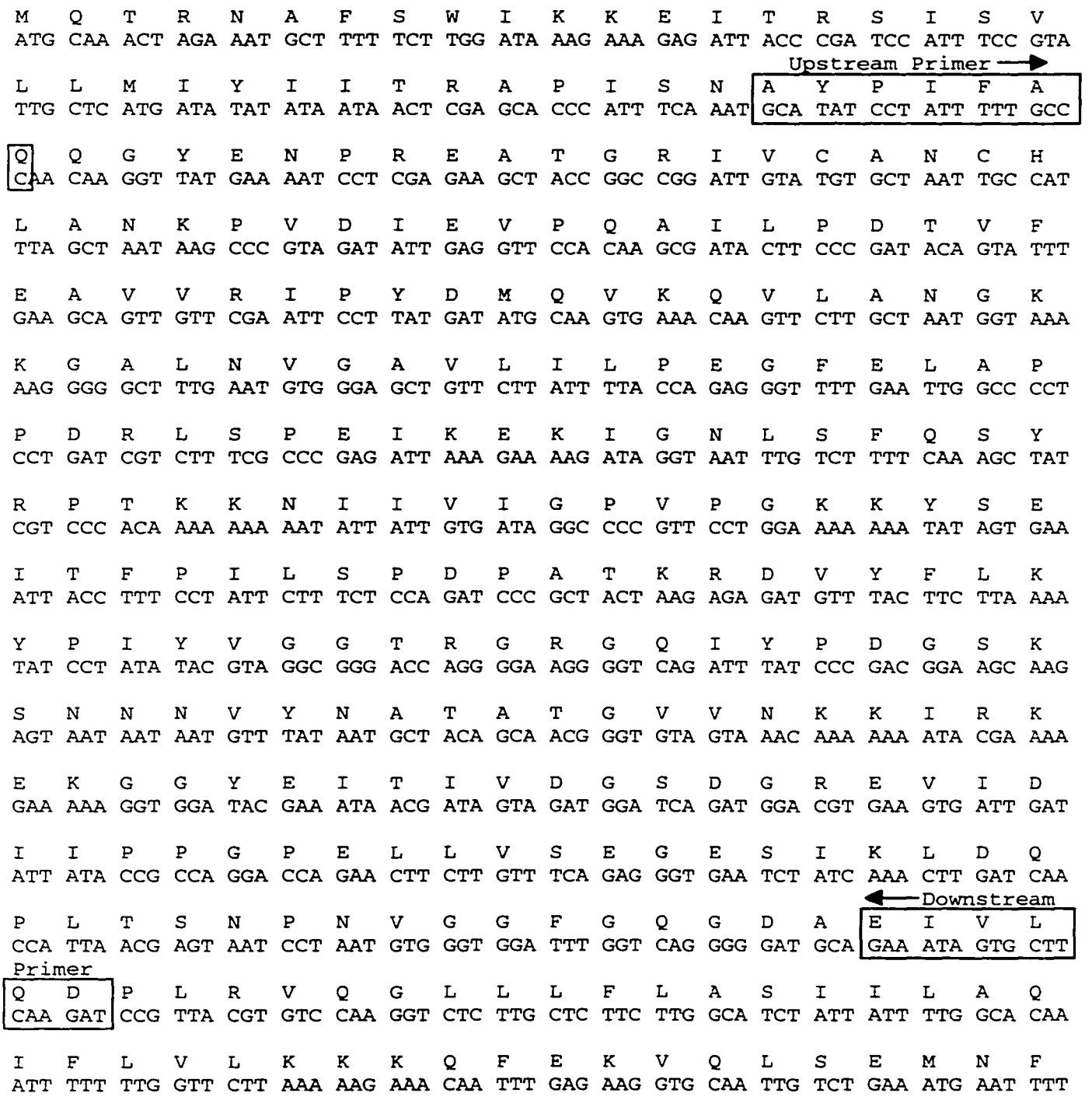
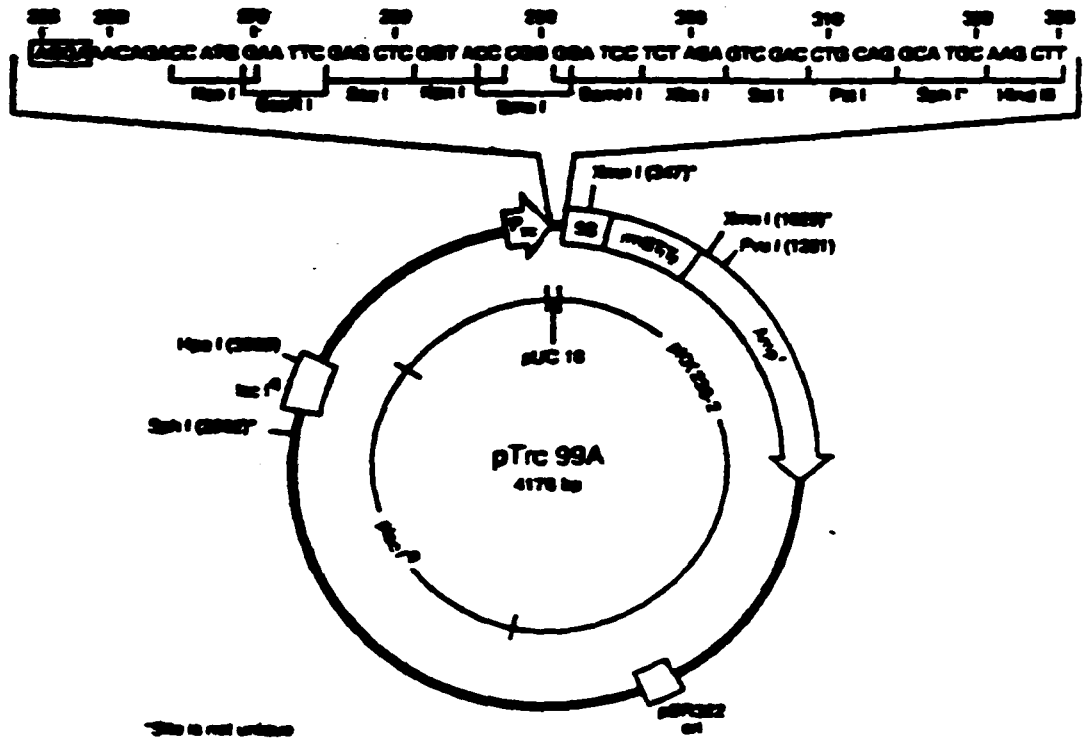

M Q T R N A F S W I K K E I T R S I S V
ATG CAA ACT AGA AAT GCT TTT TCT TGG ATA AAG AAA GAG ATT ACC CGA TCC ATT TCC GTA
Upstream Primer
L L M I Y I I T R A P I S N A Y P I F A
TTG CTC ATG ATA TAT ATA ATA ACT CGA GCA CCC ATT TCA AAT GCA TAT CCT ATT TTT GCC
Q Q G Y E N P R E A T G R I V C A N C H
CAA CAA GGT TAT GAA AAT CCT CGA GAA GCT ACC GGC CGG ATT GTA TGT GCT AAT TGC CAT
L A N K P V D I E V P Q A I L P D T V F
TTA GCT AAT AAG CCC GTA GAT ATT GAG GTT CCA CAA GCG ATA CTT CCC GAT ACA GTA TTT
E A V V R I P Y D M Q V K Q V L A N G K
GAA GCA GTT GTT CGA ATT CCT TAT GAT ATG CAA GTG AAA CAA GTT CTT GCT AAT GGT AAA
K G A L N V G A V L I L P E G F E L A P
AAG GGG GCT TTG AAT GTG GGA GCT GTT CTT ATT TTA CCA GAG GGT TTT GAA TTG GCC CCT
P D R L S P E I K E K I G N L S F Q S Y
CCT GAT CGT CTT TCG CCC GAG ATT AAA GAA AAG ATA GGT AAT TTG TCT TTT CAA AGC TAT
R P T K K N I I V I G P V P G K K Y S E
CGT CCC ACA AAA AAA AAT ATT ATT GTG ATA GGC CCC GTT CCT GGA AAA AAA TAT AGT GAA
I T F P I L S P D P A T K R D V Y F L K
ATT ACC TTT CCT ATT CTT TCT CCA GAT CCC GCT ACT AAG AGA GAT GTT TAC TTC TTA AAA
Y P I Y V G G T R G R G Q I Y P D G S K
TAT CCT ATA TAC GTA GGC GGG ACC AGG GGA AGG GGT CAG ATT TAT CCC GAC GGA AGC AAG
S N N N V Y N A T A T G V V N K K I R K
AGT AAT AAT AAT GTT TAT AAT GCT ACA GCA ACG GGT GTA GTA AAC AAA AAA ATA CGA AAA
E K G G Y E I T I V D G S D G R E V I D
GAA AAA GGT GGA TAC GAA ATA ACG ATA GTA GAT GGA TCA GAT GGA CGT GAA GTG ATT GAT
I I P P G P E L L V S E G E S I K L D Q
ATT ATA CCG CCA GGA CCA GAA CTT CTT GTT TCA GAG GGT GAA TCT ATC AAA CTT GAT CAA
Downstream
P L T S N P N V G G F G Q G D A E I V L
CCA TTA ACG AGT AAT CCT AAT GTG GGT GGA TTT GGT CAG GGG GAT GCA GAA ATA GTG CTT
Primer
Q D P L R V Q G L L L F L A S I I L A Q
CAA GAT CCG TTA CGT GTC CAA GGT CTC TTG CTC TTC TTG GCA TCT ATT ATT TTG GCA CAA
I F L V L K K K Q F E K V Q L S E M N F
ATT TTT TTG GTT CTT AAA AAG AAA CAA TTT GAG AAG GTG CAA TTG TCT GAA ATG AAT TTT

Figure 3.5. Diagram of the p*Trc* 99A expression vector (Amersham-Pharmacia) used to express a recombinant, truncated version of cytochrome *f* in *E. coli*. A portion of the *petA* gene, corresponding to the 250-amino acid hydrophilic domain of cytochrome *f*, was sub-cloned into the *Nco*I and *Hind*III restriction enzyme sites of the multiple-cloning site of the vector (shown in detail). The *Nco*I restriction enzyme site incorporated into the PCR product by the upstream primer facilitated expression of the protein fragment by providing the ATG transcription start codon necessary for transcription.


pTrc 99A
4178 bp

for 20 min. The lysate was incubated for another 20 min after the addition of 500 mM (final concentration) deoxycholate. Finally, after the addition of DNase I (to 6 μg/mL final concentration), the lysate was centrifuged for 15 min at 12,000 *g*, in an SS-34 rotor (Sorvall). The pellet was suspended in lysis buffer containing 0.5% (v/v) Triton X-100 to liberate the recombinant protein from the inclusion bodies (Sambrook et al., 1989). This suspension was then centrifuged for 15 min at 12,000 *g* and the supernatant containing the recombinant fragment of cytochrome *f* was retained for analysis.

### 3.2.6 Biochemical Analyses and Gel Filtration Chromatography

Protein measurements were performed according to Ghosh et al. (1988) as described in Chapter 2. Total lipid extracts were obtained as described in Chapter 2, and the fatty acid content of these extracts was determined by GLC after transmethylation (Ghosh et al., 1994 and Chapter 2). Size-exclusion chromatography was carried out using a column (1.6 cm i.d. x 95 cm) of Sephacryl S-300 HR (Amersham-Pharmacia) as described previously (Chapter 2).

### 3.2.7 Transmission Electron Microscopy

Floated plastoglobuli were examined by transmission electron microscopy. Specifically, a formvar-coated copper grid was placed upside-down on a droplet of freshly isolated plastoglobuli. After 1 min, the grid was drained of excess fluid and negatively stained by placing a droplet of 1% (w/v) uranyl acetate (pH 3.5) on the grid for 1 min (Katz et al., 1995; Pozueta-Romero et al., 1997). The grid was drained of excess fluid, air-dried and examined with a Phillips 300 transmission electron microscope operating at 60 kV.

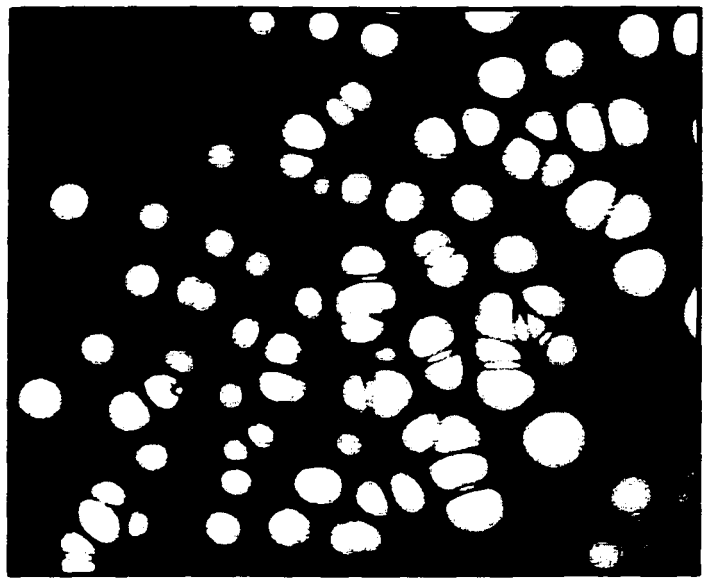
## 3.3 RESULTS

### 3.3.1 Polypeptide Composition of Plastoglobuli and Stromal Lipid-Protein Particles

Plastoglobuli were isolated from sonicated chloroplasts of *Phaseolus vulgaris* leaves by flotation centrifugation (Fig. 3.1). Transmission electron microscopy confirmed that the plastoglobuli were abundant and about 300 nm in diameter, and not contaminated with membranes (Fig. 3.6). Fractionation of washed plastoglobuli by SDS-PAGE indicated that they contain several proteins including a 32 kD polypeptide that cross-reacts with antibody raised against PAP (plastid-lipid-associated protein) from *Capsicum annuum* (Figs. 3.7A and B, lane 1). The polypeptide composition of the supernatant beneath the floated pad of plastoglobuli was also examined by SDS-PAGE. The supernatant was collected as four equal fractions (F1, F2, F3 and F4), and the protein compositions of these fractions proved to be closely similar to each other and to that of floated plastoglobuli (Fig. 3.7A, lanes 1-5). In addition, Western blot analysis of the supernatant revealed that each fraction contains the 32 kDa PAP (Fig. 3.7B, lanes 2-5). These observations collectively indicate that the supernatant contains higher-density plastoglobuli that did not float during centrifugation (Fig. 3.1). Rubisco was not detectable in gels of the supernatant fractions because Rubisco holoenzyme sediments during high-speed centrifugation for protracted periods of time (Bond, 1995).

The polypeptide composition of stromal lipid-protein particles, which were isolated from chloroplasts that had not been sonicated (Fig. 3.2), was also examined by SDS-PAGE. The protein composition of these particles was clearly distinguishable from that of both floated plastoglobuli and the higher-density plastoglobuli (Fig. 3.7A, lanes 1-6) and from that of purified thylakoid membranes (Fig. 3.7A, lanes 6 and 7). Of particular interest, though, is the

Figure 3.6. Transmission electron micrographs of plastoglobuli isolated from 2-week-old *Phaseolus vulgaris* primary leaves by flotation centrifugation and negatively stained with 1% uranyl acetate (pH 3.5). A, bar equals 500 nm; B, bar equals 250 nm.



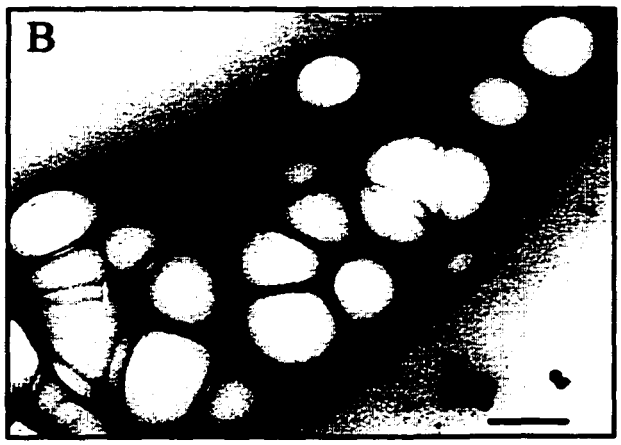
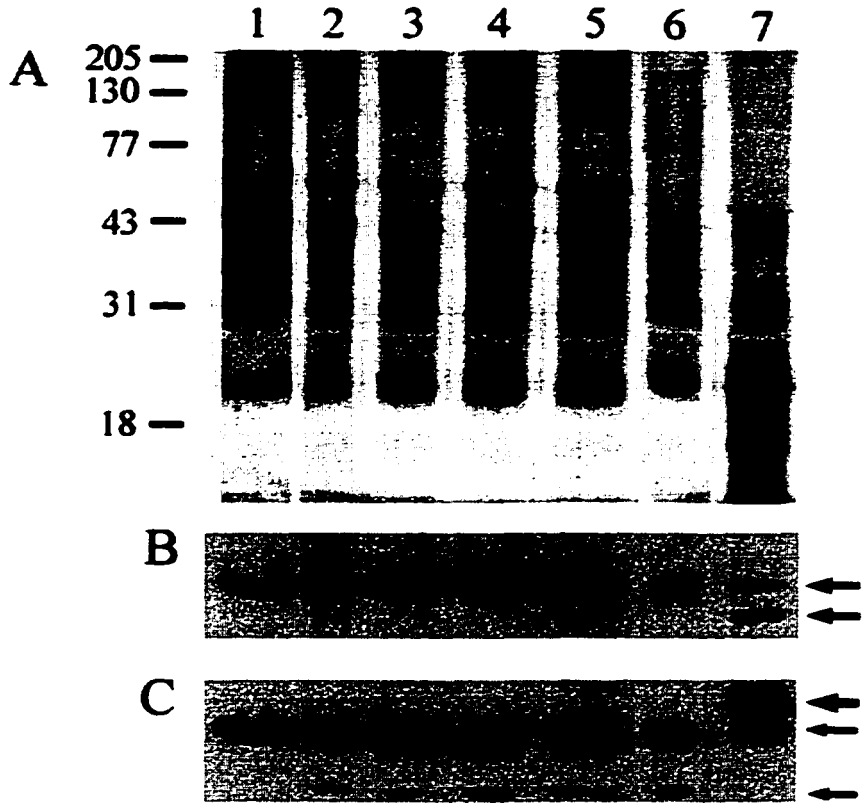

B

Figure 3.7. SDS-PAGE and Western blots of floated plastoglobuli, higher density plastoglobuli, stromal lipid-protein particles, and thylakoids. A, Silver-stained SDS-PAGE gel. Lane 1, floated plastoglobuli; lane 2, higher-density plastoglobuli (fraction F1); lane 3, higher-density plastoglobuli (fraction F2); lane 4, higher-density plastoglobuli (fraction F3); lane 5, higher-density plastoglobuli (fraction F4); lane 6, stromal lipid-protein particles; lane 7, thylakoids. Lanes were loaded with equal protein (1.2 μg). Molecular mass markers (kDa) are indicated. B, Western blot probed with plastid-lipid associated protein (PAP) antibody. Lanes are as in A. The upper arrow indicates the position of a 32 kDa protein that cross-reacts with PAP antibody. The lower arrow indicates the position of a 28 kDa protein that cross-reacts with PAP antibody. C, Western blot probed with polyclonal antibody raised against SDS-PAGE-purified cytochrome *f*. Lanes are as in A. The thick arrow indicates the position of mature, full-length cytochrome *f*. Two lower molecular weight catabolites are indicated by thin arrows.


1
2
3
4
5
6
7
A
205
130
77
43
31
18
B
C

finding that these stromal lipid-protein particles contain the 32 kDa PAP (Fig. 3.7B, lane 6), for this indicates that they are plastoglobuli-like particles.

PAP antibodies also reacted with a 32 kDa protein associated with thylakoids (Fig. 3.7B, lane 7). This protein is the same size as the PAP associated with plastoglobuli and stromal lipid-protein particles suggesting that it is a thylakoid-associated PAP (Fig. 3.7B, lanes 1-6). However, the 32 kDa PAP is clearly more abundant relative to other proteins in plastoglobuli and stromal lipid-protein particles than it is in thylakoids (Fig. 3.7B). The PAP antibodies also cross-reacted with a 28 kD polypeptide associated with thylakoids (Fig. 3.7B, lane 7). It is likely that this 28 kDa polypeptide represents another member of the PAP/fibrillin family, for all members of this protein family studied to date have very similar amino acid sequences and are likely to have common antigenic regions (Ting et al., 1998; Kessler et al., 1999; Hernández-Pinzón et al., 1999). Indeed, it has been noted previously that two members of this protein family with slightly different molecular weights are both present in chloroplasts of *Solanum tuberosum* (Eymery and Rey, 1999) and both associated with elaioplast lipid bodies (Hernández-Pinzón et al., 1999).

### 3.3.2 Association of Cytochrome *f* with Plastoglobuli and Stromal Lipid-Protein Particles

One of the characteristic features of stromal lipid-protein particles is that they contain proteolytic catabolites of certain thylakoid proteins (Ghosh et al., 1994). In light of the finding that these particles also contain PAP and, to this degree, resemble plastoglobuli, the possibility that plastoglobuli might contain thylakoid protein catabolites as well was examined. Specifically, Western blots were probed for proteolytic fragments of cytochrome *f* with antibody raised against the full-length protein. Native cytochrome *f* in thylakoids was clearly

recognized by the antibody (Fig. 3.7C, lane 7). Stromal lipid-protein particles contain two lower molecular weight polypeptides that also cross-react with cytochrome *f* antibody and hence can be presumed to be proteolytic catabolites of the native protein (Fig. 3.7C, lane 6). The largest and most abundant of these is also present in thylakoid membranes, although at a much lower level (Fig. 3.7C, lane 7), and in some cases is resolvable as two components (Fig. 3.7C, lane 6). The same catabolites of cytochrome *f* were also detectable in Western blots of the higher-density plastoglobuli (Fig. 3.7C, lanes 2-5), and the larger most abundant catabolite was discernible in floated plastoglobuli as well (Fig. 3.7C, lane 1).

In other experiments, stromal lipid-protein particles were fractionated by gel filtration chromatography on a Sephacryl column, and the proteins of the eluted fractions separated by SDS-PAGE and probed for cytochrome *f* and PAP by Western blotting. The eluted fractions were also analyzed for lipid. The finding that cytochrome *f* catabolites and PAP co-elute from the column with each other and with lipid (Fig. 3.8C) is consistent with the contention that they are all associated with the lipid-protein particles. In some of the eluted fractions, only the larger catabolite of cytochrome *f* was detectable (Fig. 3.8A, lanes 1 and 2), and in others low amounts of full-length cytochrome *f* were discernible (Fig. 3.8A, lanes 4 and 5).

### 3.3.3 Fatty Acid Composition of Plastoglobuli and Stromal Lipid-Protein Particles

The finding that floated plastoglobuli, higher-density plastoglobuli and stromal lipid-protein particles contain the same fatty acids that are found in thylakoids (Fig. 3.9) lends further support to the contention that they are derived from thylakoids. Of particular note is the fact that floated plastoglobuli and the higher-density plastoglobuli share with thylakoids the trait of having high levels (>60% of the total fatty acid complement) of linolenic acid. Indeed, the fatty acid compositions of these two plastoglobuli fractions are closely similar to each other

Figure 3.8. Immunodetection of cytochrome *f* and PAP and quantitation of fatty acid co-associated with stromal lipid-protein particles fractionated on a Sephacryl size-exclusion column. A, Western blot probed with polyclonal antibody raised against SDS-PAGE-purified mature, full-length cytochrome *f*. Lane 1, fraction 32; lane 2, fraction 33; lane 3, fraction 34; lane 4, fraction 35; lane 5, fraction 36; lane 6, fraction 37; lane 7, fraction 38. Lanes were loaded with equal volume. The thick arrow indicates the position of mature, full-length cytochrome *f*. Lower molecular weight catabolites are indicated by thin arrows. B, Western blot from A stripped of antibodies used for detection of cytochrome *f* and re-probed with PAP antibodies. The arrow indicates the position of 32 kDa PAP. Lanes are as in A. C, Total fatty acid content of pooled column fractions 31 to 34, and 35 to 38.

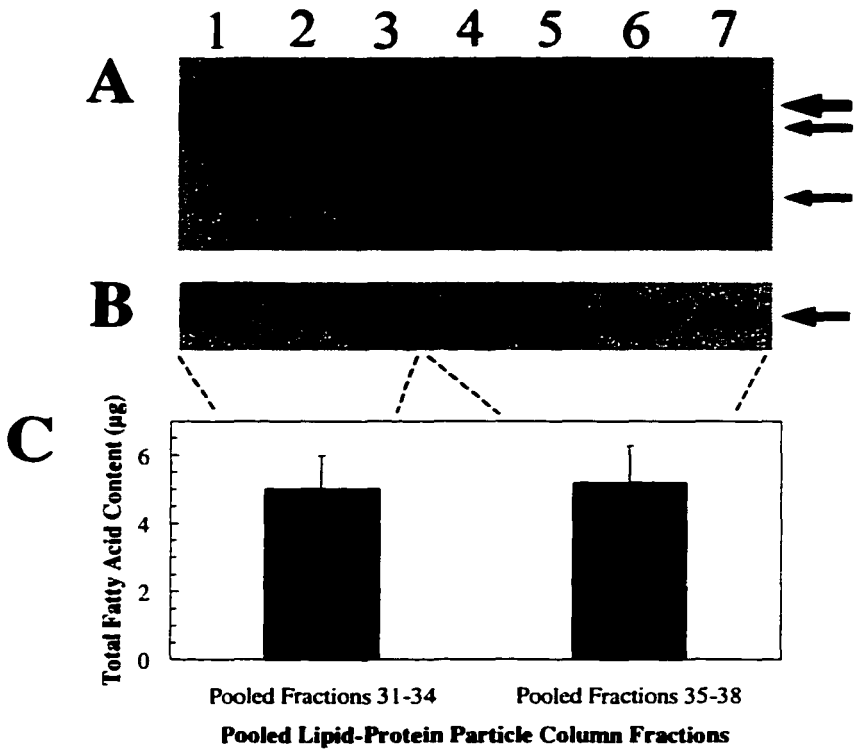
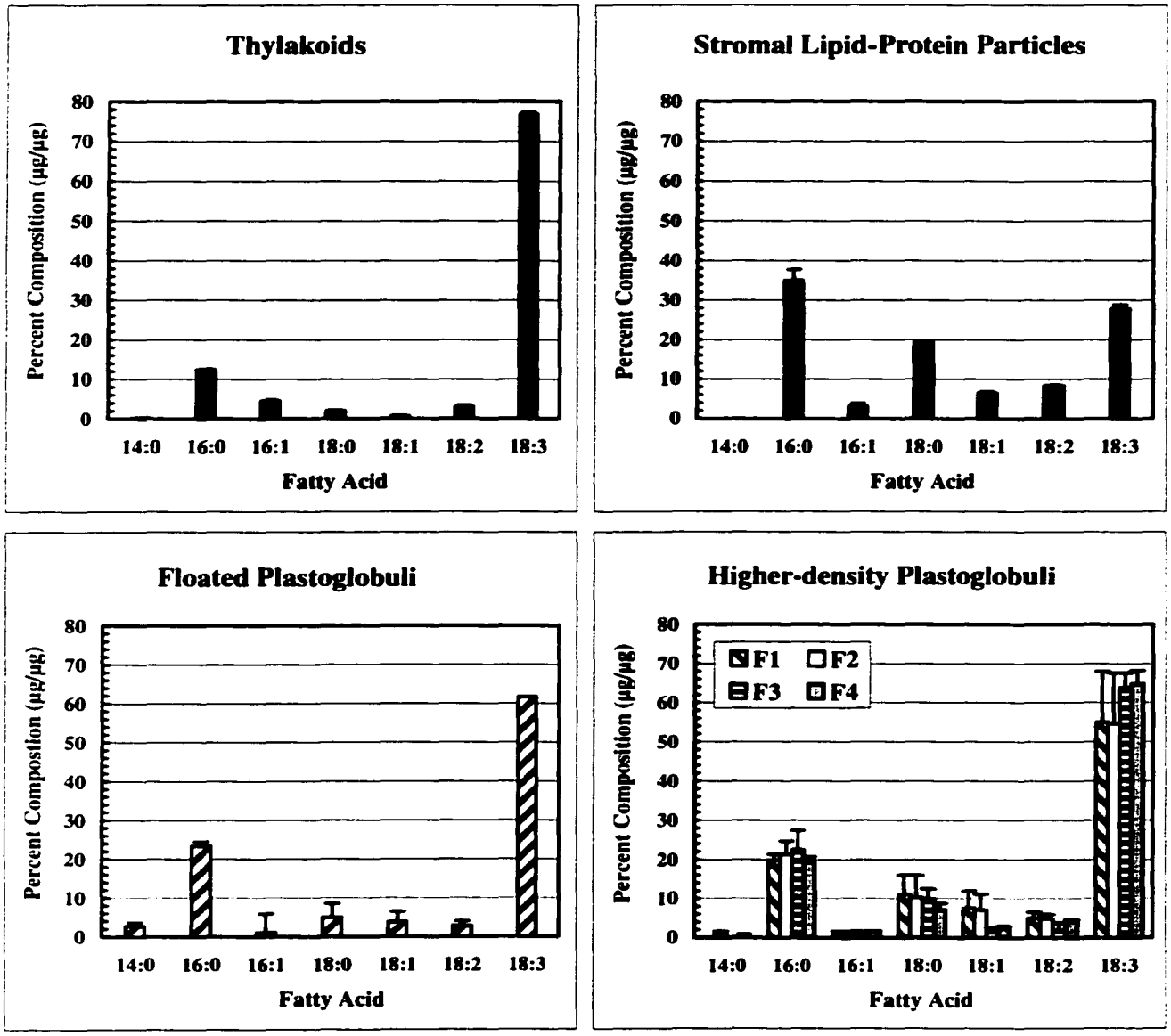

1 2 3 4 5 6 7
A
B
C
Total Fatty Acid Content (µg)
0
2
4
6
Pooled Fractions 31-34
Pooled Fractions 35-38
Pooled Lipid-Protein Particle Column Fractions

Figure 3.9. Fatty acid composition of total lipid extracts from thylakoids, stromal lipid-protein particles, floated plastoglobuli, and higher-density plastoglobuli. Values are expressed as means ± SE. 14:0, myristic acid; 16:0, palmitic acid; 16:1, palmitoleic acid; 18:0, stearic acid; 18:1, oleic acid; 18:2, linoleic acid; 18:3, linolenic acid; F1, F2, F3, and F4, higher-density plastoglobuli supernatant fractions collected sequentially from beneath the floated plastoglobuli.


Thylakoids
Percent Composition (µg/µg)
80
70
60
50
40
30
20
10
0
14:0 16:0 16:1 18:0 18:1 18:2 18:3
Fatty Acid
Stromal Lipid-Protein Particles
Percent Composition (µg/µg)
80
70
60
50
40
30
20
10
0
14:0 16:0 16:1 18:0 18:1 18:2 18:3
Fatty Acid
Floated Plastoglobuli
Percent Composition (µg/µg)
80
70
60
50
40
30
20
10
0
14:0 16:0 16:1 18:0 18:1 18:2 18:3
Fatty Acid
Higher-density Plastoglobuli
Percent Composition (µg/µg)
F1
F2
F3
F4
80
70
60
50
40
30
20
10
0
14:0 16:0 16:1 18:0 18:1 18:2 18:3
Fatty Acid

and to that of thylakoids (Fig. 3.9). However, in keeping with a previous report (Hansmann and Sitte, 1982), the plastoglobuli fractions contain higher levels of the shorter-chain fatty acid, myristic acid (14:0), than are found in thylakoids (Fig. 3.9). The fatty acid composition of stromal lipid-protein particles is clearly distinguishable from those of both plastoglobuli and thylakoids in that linolenic acid comprises only ~28% of the total fatty acid complement (Fig. 3.9).

### 3.3.4 Characterization of the Cytochrome *f* Catabolites Associated with Stromal Lipid-Protein Particles

The cytochrome *f* catabolites associated with stromal lipid-protein particles were further characterized by Western blot analysis with antibodies prepared against different regions of mature cytochrome *f*. For this purpose, polyclonal antibodies were raised against SDS-PAGE-purified mature full-length cytochrome *f* and against synthetic peptides corresponding to the C-terminus and the N-terminus of the protein. A diagrammatic representation of the topography of cytochrome *f* in the thylakoid membrane is shown in Figure 3.10, and the portions of the protein corresponding to the synthetic peptides that were used to generate polyclonal antibodies are indicated.

Before using the terminus-specific antibodies to probe thylakoids and lipid-protein particles on Western blots, the reactivity of both antisera was determined. To this end, the ability of each antiserum to react with its antigen at different dilutions was compared to that of the corresponding pre-immune serum. A comparison of the ability of cytochrome *f* N-terminus-specific antiserum and that of its corresponding pre-immune serum to react with the N-terminal peptide at a dilution of 1:1000 is shown in Figure 3.11A. The pre-immune serum does not react with the N-terminal peptide at a dilution of 1:1000. Figure 3.11A also

Figure 3.10. Schematic representation of the predicted topography of cytochrome *f* in the thylakoid membrane (adapted from Gray, 1992) and identification of the three distinct domains of the protein. The portions of the protein that correspond to the peptides that were used to generate terminus-specific antibodies are indicated with lighter shading. N, N-terminus; C, C-terminus. Numbers refer to amino acid residues.

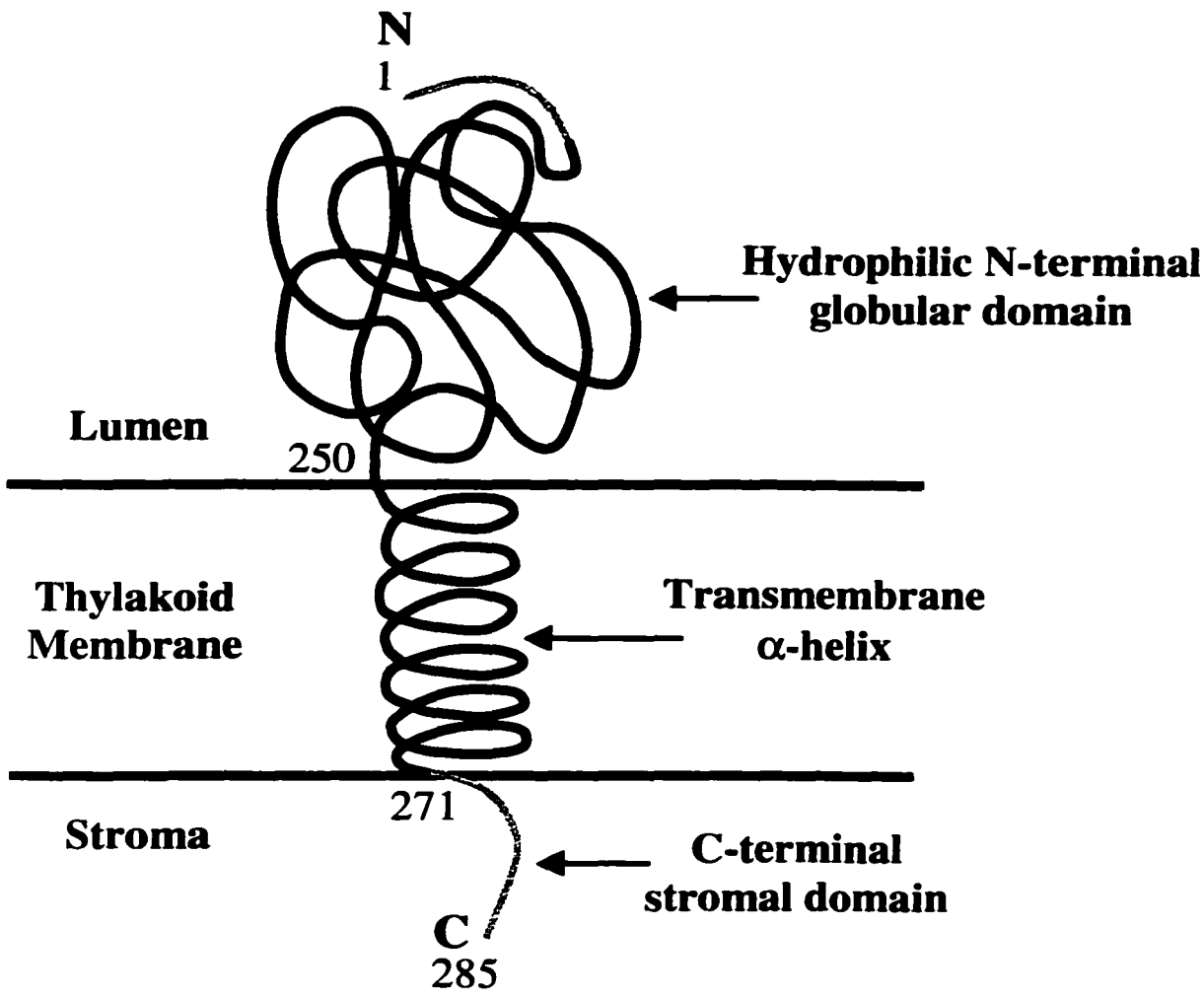
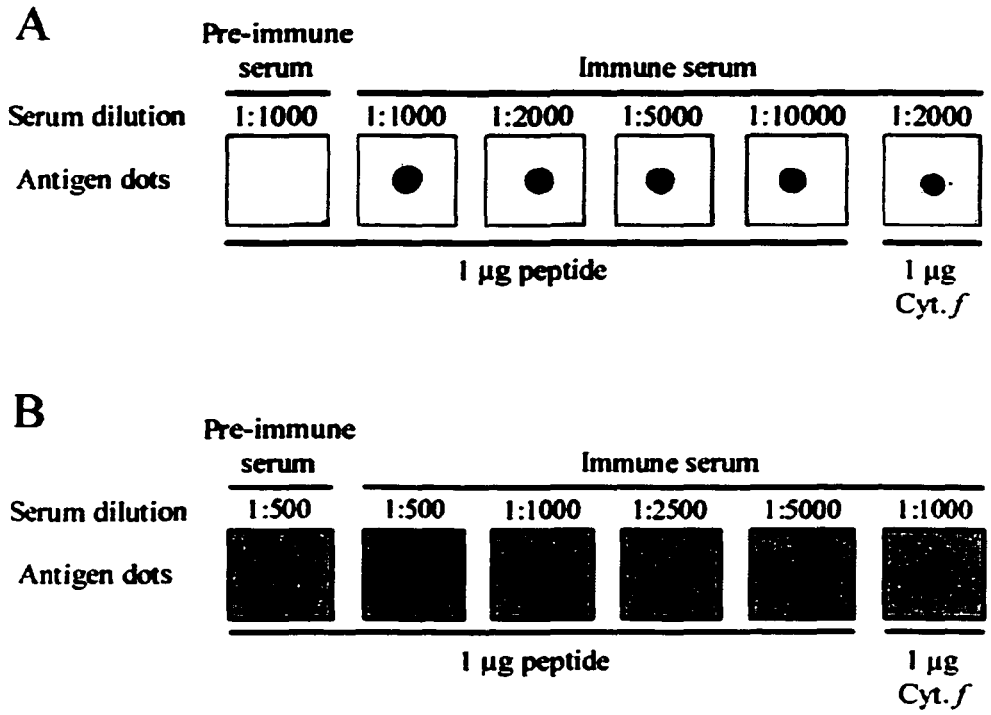

N
1
Hydrophilic N-terminal
globular domain
Lumen
250
Thylakoid
Membrane
Transmembrane
α-helix
271
Stroma
C-terminal
stromal domain
C
285

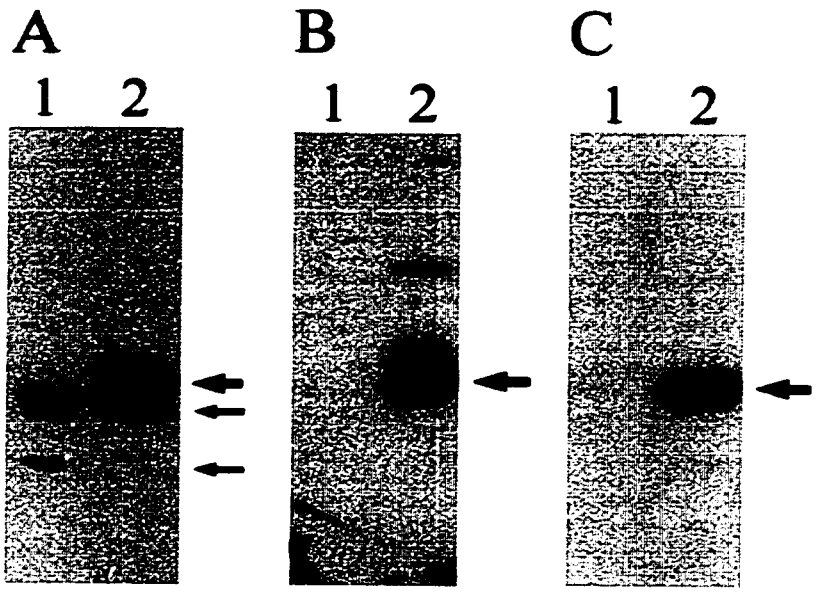
Figure 3.11. Dot-blot assays showing the titre and specificity of the peptide antibodies directed against the termini of cytochrome *f*. A, Dots of N-terminal peptide (1 μg) and full-length cytochrome *f* (1 μg) probed with various dilutions of N-terminus-specific cytochrome *f* peptide antiserum and the corresponding pre-immune serum. The colorimetric detection system was used to visualise antibody binding (Chapter 2). B, Dots of C-terminal peptide (1 μg) and full-length cytochrome *f* (1μg) probed with various dilutions of C-terminus-specific cytochrome *f* peptide antiserum and the corresponding pre-immune serum. The chemiluminescence detection system was used to visualise antibody binding.


A
Pre-immune serum
Immune serum
Serum dilution
1:1000
1:1000
1:2000
1:5000
1:10000
1:2000
Antigen dots
1 µg peptide
1 µg Cyt.f
B
Pre-immune serum
Immune serum
Serum dilution
1:500
1:500
1:1000
1:2500
1:5000
1:1000
Antigen dots
1 µg peptide
1 µg Cyt.f

demonstrates that reactivity of the N-terminus-specific antiserum declines at dilutions higher than 1:2000, and that the antiserum reacts strongly with full-length cytochrome *f* at a dilution of 1:2000. Therefore, this antibody is specific, and was used at a dilution of 1:2000 for probing Western blots. Figure 3.11B shows the comparison of the C-terminus-specific antiserum with its corresponding pre-immune serum. At a dilution of 1:500, the pre-immune serum does not react with the C-terminal peptide, whereas, the antiserum reacts strongly with the C-terminus peptide antigen at the same dilution (Fig. 3.11B). In addition, the ability of this antiserum to react with its antigen declines slightly at dilutions higher than 1:2500, and the antiserum also reacts well with full-length cytochrome *f*. Therefore, this antiserum was diluted 1:2500 when used to probe Western blots.

The polyclonal antibody raised against SDS-PAGE-purified full-length cytochrome *f* cross-reacted with mature cytochrome *f* in thylakoids (Fig. 3.12, lane 2). This antibody also recognized the largest of the lower molecular weight cytochrome *f* catabolites present in both thylakoids and stromal lipid-protein particles as well as the smaller catabolites in the lipid-protein particles (Fig. 3.12A, lanes 1 and 2). Both of the terminus-specific antibodies reacted strongly with the mature full-length protein associated with thylakoids (Figs. 3.12B, lane 2 and 3.12C, lane 2). However, neither of the terminus-specific antibodies reacted with the lower molecular weight forms of cytochrome *f* associated with thylakoids or lipid-protein particles (Figs. 3.12, B and C). These findings support the contention that the lower molecular weight forms of cytochrome *f* detectable in thylakoids and lipid-protein particles are catabolites of the mature protein. Specifically, these catabolites lack at least portions of the first 21 residues of the N-terminus and the last 15 residues of the C-terminus.

Figure 3.12. Western blots demonstrating that the lower molecular weight forms of cytochrome *f* associated with stromal lipid-protein particles and thylakoids are catabolites of the mature protein. A, Western blot probed with antibody raised against SDS-PAGE-purified full-length cytochrome *f*. Lane 1, stromal lipid-protein particles; lane 2, thylakoids. The thick arrow indicates the position of mature cytochrome *f*. Thin arrows indicate the positions of lower molecular weight catabolites. B, Western blot probed with N-terminus-specific antibody. Lanes are as in A. The arrow indicates the position of mature cytochrome *f*. C, Western blot probed with C-terminus-specific antibody. Lanes are as in A. The arrow indicates the position of mature cytochrome *f*. All lanes were loaded with equal protein (5 μg).


A
1 2
B
1 2
C
1 2

In order to determine whether the larger, abundant catabolite contains the transmembrane α-helix of cytochrome *f*, a recombinant fragment of the protein was expressed in *E. coli*. This truncated cytochrome *f* lacked the 35 C-terminal residues of the protein, including the stromal domain (15 amino acids) and the transmembrane α-helix (20 amino acids), and thus corresponded to the large hydrophilic globular N-terminus (250 amino acids) (Fig. 3.10). The relative sizes of this truncated cytochrome *f*, the mature protein and the larger, abundant catabolite were then compared by SDS-PAGE. Of particular interest is the finding that the truncated cytochrome *f* is smaller than either the native protein or the catabolite (Fig. 3.13). Since the catabolite is missing portions of both the N-terminus and the C-terminus, yet is still larger than the truncated cytochrome *f*, it must also include all or part of the transmembrane α-helix. This contention is consistent with the fact that the cytochrome *f* catabolite is associated with thylakoid membranes as well as stromal lipid-protein particles, for it is presumably anchored in the thylakoids through its transmembrane α-helix. It seems likely that the catabolite is also anchored in the lipid-protein particles through its transmembrane α-helix.

## 3.4 DISCUSSION

Two classes of plastoglobuli, those that float during protracted high-speed centrifugation and those with a higher buoyant density that remain suspended in the supernatant formed during this centrifugation, have been isolated. Both were obtained from chloroplasts that had been sonicated, a strategy designed to release plastoglobuli from thylakoids. The two classes of plastoglobuli have the same major proteins, and they also both contain the plastoglobuli-specific protein, PAP. As well, the fatty acid compositions of both types of plastoglobuli are

Figure 3.13. Western blot of a truncated recombinant form of cytochrome *f*, thylakoids and stromal lipid-protein particles. The blot was probed with antibody raised against SDS-PAGE-purified, mature, full-length cytochrome *f*. Lane 1, full-length cytochrome *f* of thylakoids (5 μg protein); lane 2, the larger, more abundant cytochrome *f* catabolite of stromal lipid-protein particles (5 μg protein); and lane 3, truncated recombinant cytochrome *f* (10 μg protein).


1
2
3

closely similar to each other and to that for thylakoids, which is consistent with the contention that they originate from thylakoids. It has been noted previously that plastoglobuli, like thylakoids, are enriched in linolenic acid (Hernández-Pinzón et al., 1999). The different buoyant densities of the two classes of plastoglobuli presumably arise from differences in protein-to-lipid ratios, possibly reflecting formation at different sites along the plane of the thylakoid membrane.

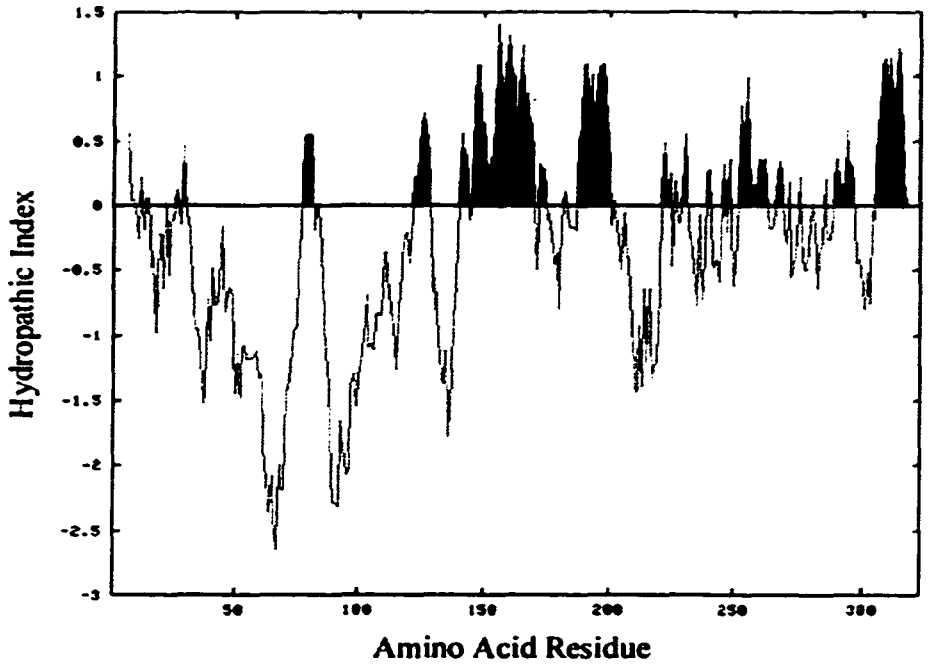
A second subpopulation of lipid particles, previously termed stromal lipid-protein particles (Ghosh et al., 1994), was isolated from chloroplasts that had not been sonicated. Earlier studies have indicated that these stromal lipid-protein particles contain galactolipids, are enriched in free fatty acids by comparison with thylakoid membranes (Chapter 2) and also contain proteolytic catabolites of thylakoid photosynthetic proteins (Ghosh et al., 1994). Indeed, it has been proposed that these particles are formed by blebbing from thylakoid membranes in much the same manner that oil bodies are formed from the endoplasmic reticulum, and that their formation allows removal of lipid and protein metabolites from the thylakoid membrane that would otherwise accumulate and destabilize its structure (Ghosh et al., 1994; Thompson et al., 1998). It is apparent from the present study that these stromal lipid-protein particles also contain the plastoglobuli-specific protein, PAP, indicating that they are generically related to plastoglobuli. Like plastoglobuli, they also originate from thylakoids as judged from the finding that they contain galactolipids as well as catabolites of thylakoid proteins (Ghosh et al., 1994). The stromal lipid-protein particles are also, however, clearly distinguishable from plastoglobuli in that they have a distinct protein composition as well as a distinct fatty acid composition, and thus they appear to be a unique class of plastoglobuli-like particles. The finding in the present study that PAP is also present in thylakoid membranes, albeit at a low relative concentration, is consistent with the contention that plastoglobuli and

stromal lipid-protein particles, which both contain this protein, are formed from thylakoid membranes.

It has been suggested that PAP may be involved in maintaining the structural integrity of chloroplastic lipid particles in much the same way that the oleosin of seed oil bodies appears to be a structural protein (Murphy, 1993; Pozueta-Romero et al., 1997; Ting et al., 1998; Eymery and Rey, 1999). Oleosin is anchored in oil bodies through a central hydrophobic domain that is embedded in the hydrophobic core of the lipid particle (Murphy, 1993). The hydropathy plot for PAP reveals hydrophobic domains that could also penetrate into the hydrophobic interior of a lipid particle (Fig. 3.14). In particular, hydropathy analysis reveals two stretches of hydrophobic residues near the center of the protein (approximately 30 residues from no. 140 to no. 170, and approximately 20 residues from no. 185 to no. 205) that could potentially be immersed in a lipid environment. Similar analyses for other members of the PAP/fibrillin family have also revealed domains that could potentially anchor the protein in a lipid matrix (Vishnevetsky et al., 1996; Ting et al., 1998). Indeed, in an earlier modeling study Knoth et al. (1986) predicted that there is a 30 kD protein component embedded in the neutral lipid matrix of plastid lipid particles.

Thylakoid proteins are thought to be voided from the membrane bilayer in association with lipid during the process of normal thylakoid turnover (Thomas and Hilditch, 1987). The finding that plastoglobuli and stromal lipid-protein particles contain lower molecular weight catabolites of cytochrome *f* raises the possibility that their formation is part of normal thylakoid turnover. Cytochrome *f* is a major protein of the thylakoid membrane and a central component of the photosynthetic electron transport chain. It is highly conserved among higher plants (see Fig. 3.3) and is anchored in the thylakoid through a single transmembrane α-helix

Figure 3.14. Hydropathy plot of the amino acid sequence of plastid-lipid-associated protein (PAP) from *Capsicum annuum* (Pozueta-Romero et al., 1997) according to the scale of Kyte and Doolittle (1982) using a window size of 11 amino acids. Hydrophobicity is indicated by positive values, and shading indicates hydrophobic domains. The analysis was performed using the ProtScale Tool available on the ExPASy Molecular Biology Server (www.expasy.ch).


Hydropathic Index
1.5
1
0.5
0
-0.5
-1
-1.5
-2
-2.5
-3
50
100
150
200
250
300
Amino Acid Residue

near the C-terminus (Gray, 1992). The 15 amino acid C-terminus (residues 271-285) extends into the stroma, and the large globular N-terminus (residues 1-250) protrudes into the thylakoid lumen (Fig. 3.10) (Gray, 1992). The lower molecular weight catabolites of cytochrome *f* were not recognized by antibodies raised against synthetic peptides corresponding to the C-terminus and N-terminus of the full-length protein. This indicates that the catabolites are formed as a result of proteolytic cleavage at both ends of the protein, events that presumably render the protein non-functional causing it to be voided from the thylakoid. There would appear to be a protease capable of cleaving cytochrome *f* associated with thylakoids (Gray, 1992). Indeed, the larger cytochrome *f* catabolite is actually discernible in thylakoids, albeit at a lower concentration than in the lipid particles, supporting the contention that at least this catabolite is formed on the thylakoid membrane and subsequently voided.

Several lines of evidence indicate that the lower molecular weight catabolites of cytochrome *f* detectable in isolated plastoglobuli and stromal lipid-protein particles are native components of the particles and not simply free polypeptides. First, the cytochrome *f* catabolites associated with lipid-protein particles co-elute stoichiometrically during size-exclusion column chromatography of the particles, indicating that they are co-associated rather than free polypeptides. Second, the catabolites co-elute with lipid, which indicates that they are eluting as elements of lipid particles rather than as free polypeptides. Third, the cytochrome *f* catabolites were only detectable in a subset of the lipid-protein particles eluted from the size-exclusion column. This indicates that they are not simply free polypeptides adhering to the surface of the particles as contaminants, for if this were the case they would either be randomly associated with eluted particles or present in all of the eluted fractions. Fourth, at least one of the cytochrome *f* catabolites, the larger one, contains the transmembrane

α-helix of the native protein, which is presumably embedded in the interior of the lipid particle. Indeed, when lipid-protein particles were fractionated by size-exclusion chromatography, some of the separated particles were found to contain small amounts of the full-length cytochrome *f* protein as well as its catabolites, and they all co-eluted indicating that they were co-associated. The full-length cytochrome *f* is presumably also anchored to the lipid-protein particles through its transmembrane α-helix. Full-length versions of thylakoid photosynthetic proteins have been detected previously in stromal lipid-protein particles and are thought to be denatured proteins that are no longer functional and, accordingly, have been voided from the membrane bilayer (Ghosh et al., 1994). Finally, the cytochrome *f* catabolites also co-elute with PAP during size-exclusion chromatography of lipid-protein particles indicating that they are co-associated with this protein as well. Since PAP and other members of the PAP/fibrillin protein family are known to be associated with plastid lipid particles, this also supports the contention that the cytochrome *f* catabolites are native elements of plastoglobuli and stromal lipid-protein particles.

It is possible, therefore, that the genesis of both plastoglobuli and stromal lipid-protein particles is an inherent feature of thylakoid turnover, allowing removal of cytochrome *f* and perhaps other photosynthetic proteins that have been proteolytically cleaved or otherwise damaged. These two subpopulations of chloroplast lipid particles appear to be generically related in that they both contain the plastoglobuli-specific protein, PAP. Indeed, it seems reasonable to classify stromal lipid-protein particles as plastoglobuli-like particles. However, plastoglobuli and stromal lipid-protein particles are also distinguishable. In particular, they have different polypeptide and fatty acid compositions. Yet, both are clearly derived from the thylakoid membrane, and these differences may simply reflect different points of origin along

the plane of the thylakoid membrane. Distinguishable populations of plastoglobuli have also been isolated recently from chloroplasts of *Pisum sativum* on a sucrose gradient (Kessler et al., 1999). Given their prospective role in thylakoid turnover, it is conceivable that the genesis of plastoglobuli and plastoglobuli-like particles is also involved in thylakoid repair, and even the dismantling of thylakoids, following episodes of environmental stress or during certain stages of development by facilitating the removal of damaged molecules. It has been reported, for example, that members of the PAP/fibrillin family are upregulated in response to drought (Chen et al., 1998; Eymery and Rey, 1999) and in embryos of mid-cotyledonary stage *Brassica napus* when thylakoids are being dismantled (Hernández-Pinzón et al., 1999).

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# 4. Identification of a Lipase from *Phaseolus vulgaris* Leaves that is Up-Regulated During Senescence

## 4.1 INTRODUCTION

The enzymes responsible for lipid catabolism are generally referred to as lipases. These lipolytic enzymes are classified based on the lipid substrate that they degrade and their general site of cleavage. For example, different classes of phospholipases have been identified which degrade phospholipids at one of a number of possible cleavage sites (Chapman, 1998). True lipases (triacylglycerol acyl hydrolases) are defined biochemically as enzymes that attack triacylglycerols at an oil-water interface, liberating free fatty acids (Galliard, 1980). Galactolipases and some phospholipases, on the other hand, belong to a group of enzymes termed lipolytic acyl hydrolases (Galliard, 1980). Members of this group also deacylate their lipid substrate. However, their substrates include polar membrane lipids (e.g. galactolipids, phospholipids), rather than triacylglycerols. Non-specific lipolytic acyl hydrolases are known to degrade a wide range of lipids including non-polar diacylglycerol (Galliard, 1980). Lipolytic enzymes of all kinds are involved in signaling cascades, membrane reorganization, membrane degradation, neutral lipid mobilization during seed germination and perhaps act as virulence factors in pathogens (Jaeger et al., 1994; Brick et al., 1995; Chapman, 1998; Wang, 1999). Lipase will be used here as a general term to refer to any enzyme that attacks a lipid substrate, unless otherwise indicated.

Sequence analysis has revealed a semi-conserved pentapeptide, G-X-S-X-G (where X represents any amino acid) in lipases cloned from animals, bacteria, fungi and plants

(Derewenda and Derewenda, 1991; Brick et al., 1995). The serine residue of this lipase consensus sequence is part of the active site of the enzyme (Derewenda and Derewenda, 1991). In addition, analysis of triacylglycerol lipases from plants has revealed a ten-amino acid semi-conserved peptide, [LIV]-X-[LIVFY]-[LIVNST]-G-[HYWV]-S-X-G-[GSTAC], that includes the lipase consensus pentapeptide.

Lipases play critical roles in the turnover of membrane lipids and the breakdown of lipids during plant senescence (Douce and Joyard, 1980; Galliard, 1980). It is well documented, for example, that membrane leakage due to lipid degradation and leading to a loss of intercellular compartmentalization is a critical step in senescence (Ferguson and Simon, 1973; Barber and Thompson, 1980; Thompson, 1984). In addition, lipases are thought to play a key role in the chilling sensitivity of some plants (Kaniuga et al., 1999). It is surprising, therefore, that specific roles for lipid-degrading enzymes in turnover and senescence have not yet been fully elucidated. While lipases from mammalian systems have been extensively studied, research on their plant counterparts has lagged behind. Indeed, as recently as 1980, knowledge of lipolytic plant enzymes was referred to as "meager" (Galliard, 1980). As an example, only recently was the first phospholipase D gene from plants cloned (Wang et al., 1994). As well, lipases from leaves, specifically galactolipases and other lipases active in chloroplasts, while long suspected as being important enzymes for lipid turnover and senescence, have received relatively little attention in the literature. Galactolipases from the chloroplasts of *Phaseolus* and other higher plants have been isolated and partially characterized on a biochemical level (Sastry and Kates, 1964; Helmsing, 1969; Anderson et al., 1974; Matsuda et al., 1979). However, a gene for such an enzyme has not yet been isolated.

A gene encoding a lipase from carnation petal cytosol has recently been cloned. Northern blot analysis showed that the expression of this gene is up-regulated during senescence and following exposure of mature flowers to exogenous ethylene or treatment with abscisic acid (J.E. Thompson, personal communication). Therefore, it is believed that this enzyme plays a critical role in carnation petal senescence. The clone was isolated by screening a cDNA expression library made from the mRNA of senescing carnation petals with antibodies raised against lipid-protein particles isolated by flotation from the cytosol of the same petals. Therefore, it seems possible that this enzyme may associate with cytosolic lipid-protein particles as well as membranes. The deduced amino acid sequence for the carnation lipase clone is shown in Figure 4.1. The association of lipases with lipid bodies isolated from *Pinus edulis*, *Brassica napus* and *Zea mays* has been reported previously (Lin et al., 1983; Hammer and Murphy, 1993; Hoppe and Theimer, 1997).

Lipid-protein particles from the stroma of chloroplasts are similar to those isolated from cytosol. Like their cytosolic counterparts, chloroplastic lipid-protein particles are spherical and osmiophilic. They have been shown to contain thylakoid proteins and their catabolites as well as lipids and lipid catabolites of thylakoid origin (Ghosh et al., 1994; Chapter 3). The particles also contain the same fatty acids as thylakoids, although the fatty acids are found in different relative proportions. Furthermore, the lipid-protein particles have been found to contain relatively more free fatty acids than thylakoids (Ghosh et al., 1994; Chapter 3). It has, therefore, been suggested that the particles are derived from thylakoids and are involved in turnover of the membrane (Ghosh et al., 1994; Thompson et al., 1998; Chapter 3).

The purpose of this study was to determine whether one or more lipase genes are up-regulated in senescing leaves. The study began with an attempt to clone a lipase cDNA from

Figure 4.1. Nucleotide and corresponding amino acid sequence of a lipase cDNA clone (Accession No. AAD01804; Hong et al., direct submission) isolated from carnation (*Dianthus caryophyllus*) petal cytosol. The clone was used as a heterologous probe to detect a lipase gene expressed in the primary leaves of *Phaseolus vulgaris* using Northern blot analysis. The lipase consensus sequence (pentapeptide) is highlighted (G-X-S-X-G).

```
M   A   A   E   A   Q   P   L   G   L   S   K   P   G   P   T   W   P   E   L   L   G
ATG GCT GCA GAA GCC CAA CCT TTA GGC CTC TCA AAG CCC GGC CCA ACA TGG CCC GAA CTC CTC GGG

S   N   A   W   A   G   L   L   N   P   L   N   D   E   L   R   E   L   L   L   R   C
TCC AAC GCT TGG GCC GGG CTA CTA AAC CCG CTC AAC GAT GAG CTC CGT GAG CTC CTC CTA CGC TGC

G   D   F   C   Q   V   T   Y   D   T   F   I   N   D   Q   N   S   S   Y   C   G   S
GGG GAC TTC TGC CAG GTG ACA TAC GAC ACC TTC ATA AAC GAC CAG AAC TCG TCC TAC TGC GGC AGC

S   R   Y   G   K   A   D   L   L   H   K   T   A   F   P   G   G   A   D   R   F   D
AGC CGC TAC GGG AAG GCG GAC CTA CTT CAT AAG ACC GCC TTC CCG GGG GGC GCA GAC CGG TTT GAC

V   V   A   Y   L   Y   A   T   A   K   V   S   V   P   E   A   F   L   L   K   S   R
GTG GTG GCG TAC TTG TAC GCC ACT GCG AAG GTC AGC GTC CCA GAG GCG TTT CTG CTG AAG TCG AGG

S   R   E   K   W   D   R   E   S   N   W   I   G   Y   V   V   V   S   N   D   E   T
TCG AGG GAG AAG TGG GAT AGG GAA TCG AAT TGG ATT GGG TAT GTC GTG GTG TCG AAT GAC GAG ACG

S   R   V   A   G   R   R   E   V   Y   V   V   W   R   G   T   C   R   D   Y   E   W
AGT CGG GTG GCG GGA CGA AGG GAG GTG TAT GTG GTG TGG AGA GGG ACT TGT AGG GAT TAT GAG TGG

V   D   V   L   G   A   Q   L   E   S   A   H   P   L   L   R   T   Q   Q   T   T   H
GTT GAT GTT CTT GGT GCT CAA CTT GAG TCT GCT CAT CCT TTG TTA CGC ACT CAA CAA ACT ACT CAT

V   E   K   V   E   N   E   E   K   K   S   I   H   K   S   S   W   Y   D   C   F   N
GTT GAA AAG GTG GAA AAT GAG GAA AAG AAG AGC ATT CAT AAA TCA AGT TGG TAC GAC TGT TTC AAT

I   N   L   L   G   S   A   S   K   D   K   G   K   G   S   D   D   D   D   D   D   D
ATC AAC CTA CTA GGT TCC GCG TCC AAA GAC AAA GGA AAA GGA AgC GAC GAC GAC GAT GAT GAC GAC

P   K   V   M   Q   G   W   M   T   I   Y   T   S   E   D   P   K   S   P   F   T   K
CCC AAA GTG ATG CAA GGT TGG ATG ACA ATA TAC ACA TCG GAG GAT CCC AAA TCA CCC TTC ACA AAA

L   S   A   R   T   Q   L   Q   T   K   L   K   Q   L   M   T   K   Y   K   D   E   T
CTA AGT GCA AGA ACA CAA CTT CAG ACC AAA CTC AAA CAA CTA ATG ACA AAA TAC AAA GAC GAA ACC

L   S   I   T   F   A   G   H   S   L   G   A   T   L   S   V   V   S   A   F   D   I
CTA AGC ATA ACA TTC GCC GGT CAC AGC CTA GGC GCG ACA CTA TCA GTC GTG AGC GCC TTC GAC ATA

V   E   N   L   T   T   E   I   P   V   T   A   V   V   F   G   C   P   K   V   G   N
GTG GAG AAT CTC ACG ACC GAG ATC CCA GTC ACG GCC GTG GTC TTC GGG TGC CCA AAA GTA GGC AAC

K   K   F   Q   Q   L   F   D   S   Y   P   N   L   N   V   L   H   V   R   N   V   I
AAA AAA TTC CAA CAA CTC TTC GAC TCG TAC CCA AAC CTA AAT GTC CTC CAT GTA AGG AAT GTC ATC

D   L   I   P   L   Y   P   V   K   L   M   G   Y   V   N   I   G   I   E   L   E   I
GAC CTG ATC CCT CTG TAT CCC GTG AAA CTC ATG GGT TAC GTG AAC ATA GGA ATC GAG CTG GAG ATC

D   S   R   K   S   T   F   L   K   D   S   K   N   P   S   D   W   H   N   L   Q   A
GAC TCG AGG AAG TCG ACC TTT CTA AAG GAC TCG AAA AAC CCG AGT GAT TGG CAT AAT TTG CAA GCA

I   L   H   V   V   S   G   W   H   G   V   K   G   E   F   K   V   V   N   K   R   S
ATA TTG CAT GTT GTA AGT GGT TGG CAT GGG GTT AAG GGG GAG TTT AAG GTT GTA AAT AAG AGA AGT

V   A   L   V   N   K   S   C   D   F   L   K   E   E   C   L   V   P   P   A   W   W
GTT GCA TTG GTT AAT AAG TCA TGT GAT TTT CTT AAG GAA GAA TGT TTG GTT CCT CCA GCT TGG TGG

V   V   Q   N   K   G   M   V   L   N   K   D   G   E   W   V   L   A   P   P   E   E
GTT GTG CAG AAC AAA GGG ATG GTT TTG AAT AAG GAT GGT GAG TGG GTT TTG GCT CCT CCT GAG GAA

D   P   T   P   E   F   D
GAT CCT ACT CCT GAA TTT GAT
```

senescing *Phaseolus vulgaris* leaves. Cloning of a leaf lipase gene that is up-regulated during senescence would be a significant breakthrough toward gaining a more complete understanding of membrane degradation during leaf senescence.

## 4.2 MATERIALS AND METHODS

### 4.2.1 Plant Material

*Phaseolus vulgaris* cv. Kinghorn Wax (yellow wax bean) seedlings were grown in Pro-Mix BX (Premier Brands, Red Hill, PA) in 9-inch pots under greenhouse conditions supplemented with a 16-h photoperiod of light from a high-pressure sodium lamp. Each pot containined twenty seedlings, and primary leaves were harvested 1, 2, and 3 weeks after planting. Some pots of 2-week old seedlings were treated with 1 ppm ethylene in a sealed chamber for 12-16 hours before harvesting.

### 4.2.2 Biochemical Analyses

Extracts were prepared from primary leaves for total protein and chlorophyll measurements. Approximately 5 g (fresh weight) of primary leaves from 1-, 2-, and ethylene treated 2-week-old seedlings, and 10 g of primary leaves from 3-week-old seedlings were frozen in liquid nitrogen and ground to a fine powder using a mortar and pestle. The powder was added to 25 mL of buffer A, and the homogenate was filtered through 2 layers of miracloth. The filtrate was used to measure total protein and chlorophyll content of the leaves as described in Chapter 2.

### 4.2.3 Analysis of RNA

All glassware used in the extraction of RNA was baked overnight at 200°C to denature any contaminating RNases prior to use. As well, sterile, disposable, RNase-free plasticware was used wherever possible. All water used during the procedure was pre-treated with 1% (v/v) diethyl pyrocarbonate (DEPC) according to Sambrook et al. (1989). DEPC inactivates RNases, and the DEPC-treated water was used to make all buffers used for RNA extraction.

#### *4.2.3.1 Extraction of Total RNA from Leaves*

Total RNA was extracted from leaves essentially as described by Davis et al. (1986). Primary leaves were frozen immediately upon harvesting using liquid nitrogen, and either stored at –70°C until use or ground to a powder immediately using a mortar and pestle. Approximately 5 g (fresh weight) of 1-, 2-, and ethylene-treated 2-week tissue, and approximately 10 g (fresh weight) of 3-week tissue were used for extraction of total RNA. The powder was added immediately to 25 mL of freshly prepared guanidine isothiocyanate buffer (4 M guanidine isothiocyanate, 25 mM sodium acetate [pH 6.0], and 120 mM β-mercaptoethanol) to inactivate any RNases contained in the sample. The homogenate was then filtered through two layers of miracloth into autoclaved 30 mL screw-cap centrifuge tubes. The filtrate was centrifuged for 30 min at 27,000 *g* in an SS-34 rotor (Sorvall). The pellet from this centrifugation was discarded. The supernatant was removed and overlaid onto a 10 mL cushion of 5.7 M CsCl in disposable 35 mL Ultra-Clear thinwall centrifuge tubes (Beckman) and centrifuged at 10°C for 17-21 hours at 130,000 *g* in an SW-28 Ti rotor (Beckman). The upper layer was removed by aspiration using a sterile, disposable pipette, and discarded. The remaining CsCl layer was decanted, and the small translucent, jelly-like RNA pellet rinsed

briefly with ice-cold RNase-free 70% ethanol. The pellet was then redissolved in 500 µL of DEPC-treated water and 100 µL of 3 M sodium acetate (pH 5.2), and transferred to an autoclaved RNase-free 1.5 mL microcentrifuge tube. The total RNA extract was then further purified by phenol extraction. This was achieved by adding 300 µL phenol and 300 µL chloroform (each designated for RNA use only) to the RNA suspension. The mixture was then mixed well and centrifuged for 4 min at maximum speed (16,500 *g*) in a microcentrifuge. The upper layer was removed by aspiration, and 600 µL chloroform was added to the remaining solution. The contents of the tube were then mixed well and centrifuged again for 4 min at 16,500 *g*. The upper layer was removed and divided into two 300 µL aliquots. After addition of 900 µL of ice-cold absolute ethanol to each, the aliquots of total RNA were stored at –70°C in autoclaved 1.5 mL microcentrifuge tubes for at least 2 h before use.

#### *4.2.3.2 Preparation of RNA and Formaldehyde-Agarose Gel for Northern Blot Analysis*

Total RNA was precipitated immediately prior to Northern blot analysis. The RNA extract that was suspended in DEPC-treated water, sodium acetate and ethanol was removed from –70°C and centrifuged at 4°C for 30 min at maximum speed in a microcentrifuge. The resulting RNA pellet was resuspended in a small volume of DEPC-treated water, and its absorbance was read at 260 nm in a spectrophotometer (Beckman model DU-64) after a 50-fold dilution in DEPC water. The concentration of RNA was determined using the following equation: $A_{260} = 1 = 40$ µg RNA·mL$^{-1}$ (Sambrook et al., 1989). An aliquot equivalent to 10 µg of total RNA (in a total volume of 10 µL or less) was removed for Northern blotting. The remainder of the precipitated RNA was stored at –70°C in aliquots containing 10 µg total RNA each, for future use. To prepare the RNA sample for electrophoresis, 20 µL of RNA

denaturation buffer (containing [per 1.32 mL] 750 µL formamide, 150 µL 10-times MOPS buffer [200 mM MOPS, 50 mM sodium acetate and 10 mM EDTA, pH 7.0], 240 µL formaldehyde, 100 µL DEPC-treated water, and 80 µL 10% (w/v) bromophenol blue) and 0.5 µL ethidium bromide (0.5 µg/µL) were added to the 10 µg aliquot of total RNA.

To prepare the formaldehyde-agarose gel used for electrophoresis of the RNA, 0.5 mg of RNase-free agarose was suspended in 41 mL of single-strength MOPS buffer and dissolved by heating the mixture in a microwave. After allowing the dissolved agarose to cool to about 60°C, 9 mL of formaldehyde was added with constant mixing. The solution was then poured into a small gel tray with a six-well comb in place, and allowed to polymerize at room temperature. The gel tray and buffer chamber used for electrophoresis of RNA were soaked in DEPC-treated water overnight prior to use. After polymerization, the entire gel apparatus containing the gel as well as the electrophoresis running buffer (single-strength MOPS buffer) was placed at 4°C for at least 30 min prior to running the gel.

The RNA in denaturing solution was incubated for 5 min at 65°C and placed directly on ice immediately prior to loading onto the gel. After loading an equal amount (10 µg) of each RNA extract, the gel was run for 10 min at 210 V. The voltage was then adjusted to 80 V, and the gel was run at this voltage for an additional 1.5 h.

#### *4.2.3.3 Transfer of RNA to Nylon Membrane*

Upon completion of electrophoresis, an image of the gel, illuminated using UV irradiation, was captured using a Pharmacia Image Master VDS equipped with a digital camera and connected to a computer. The RNA was then transferred from the gel to a nylon membrane (BIOTRANS, ICN Biomedicals) by capillary action using 6-times SSC buffer (containing [per

litre] 52.6 g NaCl and 26.5 g sodium citrate, pH adjusted to 7.0 with NaOH) overnight according to Sambrook et al. (1989) and the membrane manufacturer's instructions (ICN Biomedicals). Specifically, the gel was placed upside down on a piece of saturated filter paper, which was in turn placed over a solid support. The filter paper extended over the edges of the solid support into the reservoir of 6-times SSC transfer buffer. The gel was then surrounded by strips of parafilm to ensure that transfer solution only passed through the gel and not around it. A piece of nylon membrane was placed on top of the gel so that it completely covered the surface of the gel, and two pieces of filter paper were placed over the membrane. Air bubbles were then removed by rolling a clean glass pipette over the surface. A stack of 8-10 cm of paper towel was then placed on top, followed by a glass plate and a 1 kg weight. The transfer was allowed to proceed overnight. After transfer, the RNA was cross-linked to the damp nylon membrane using UV irradiation in a Bio-Rad GS Gene Linker UV Chamber (setting C3 for wet membranes).

#### *4.2.3.4 Preparation of Radiolabeled DNA Probe*

The RNA blot was probed with a heterologous cDNA clone corresponding to a lipase gene isolated from carnation petal cytosol (Fig. 4.1) that was radiolabeled with $^{32}P$. The radioactive $^{32}P$ was incorporated into the DNA using a random-primed DNA labeling kit according to the manufacturer's instructions (Boehringer Mannheim/Roche). Specifically, 100 ng of DNA (isolated full-length carnation lipase cDNA; kindly provided by M. Wang and J.E. Thompson, Department of Biology, University of Waterloo) in 10 µL water was denatured by heating for 5 min at 100°C and subsequently cooled on ice. One µL each of dATP, dGTP and dTTP, and 2 µL of the hexanucleotide reaction mixture (containing random hexamers and 10-times

concentrated buffer) were then added to the denatured DNA. Working in a fume hood behind a plexi-glass shield, 4 μL of [$\alpha^{32}$P] dCTP (~50 μCi) and 1 μL of Klenow fragment DNA polymerase were added, and the mixture was incubated at 37°C for 30 min. The reaction was stopped by the addition of 80 μL of TE buffer (10 mM Tris (pH 8.0), 1 mM EDTA). Non-incorporated dNTPs were then removed using a small (~0.9 mL) Sephadex G-50 column equilibrated using TE buffer. The amount of label incorporated into the DNA was determined by diluting a 1 μL aliquot of the aqueous reaction mixture in EcoLite liquid scintillation fluid and measuring radioactivity using a liquid scintillation counter (Beckman model LS7000). A volume of labeled probe corresponding to 1 x $10^7$ CPM was then used to probe the RNA on a Northern blot.

#### *4.2.3.5 Hybridization of Radiolabeled DNA Probe with RNA*

The RNA cross-linked to the nylon membrane was incubated at 42°C for at least 2 hours prior to the addition of the radio-labeled DNA probe in 10 mL of pre-hybridization solution (4 mL formamide, 3 mL 20-times concentrated SSC, 1 mL 50-times concentrated Denhardt's solution, 500 μL 10% [w/v] SDS, 1.3 mL dd$H_2O$, and 200 μL salmon sperm DNA [10 mg/mL] denatured by heating for 10 min at 100°C) in a large hybridization tube with constant mixing in a hybridization oven (VWR Scientific, model 2720). The radiolabeled DNA probe was added to the prehybridization solution and allowed to hybridize with the nylon membrane-bound RNA overnight, while gently mixing in the hybridization oven at 42°C.

Following hybridization, the solution containing the radiolabeled probe was discarded as radioactive liquid waste, and the nylon membrane was washed for 5 min at room temperature with double-strength SSC containing 0.1% (w/v) SDS. The membrane was then washed for 30

min at 42°C with double-strength SSC containing 0.1% (w/v) SDS in the hybridization oven. Following this wash, the membrane was placed on a piece of 3 MM Whatman blotting paper and wrapped in plastic wrap. Working in a darkroom, the membrane was exposed to autoradiography film (Kodak XL-1 Blue) and secured in place in a light-tight autoradiography exposure cassette between two autoradiographic intensifying screens. Exposure was continued overnight at minus 70°C. The resulting autoradiogram was developed in a dark room using conventional film developing solutions and techniques.

### 4.2.4 Isolation of a Lipase Clone from *Phaseolus* Leaves

#### *4.2.4.1 Screening of a cDNA Library*

An attempt was made to isolate a lipase cDNA clone from *Phaseolus vulgaris* primary leaves. This involved screening a cDNA library constructed from the mRNA of mature (~2 week-old) primary leaves of *Phaseolus vulgaris* seedlings with the heterologous full-length cDNA carnation lipase clone. The library was constructed by Dr. Yuwen Hong in the laboratory of Dr. Shimon Gepstein (Technion-Israel Institute of Technology, Haifa, Israel). However, the cDNA clones that were isolated from the bean leaf library did not correspond to a lipase when compared to protein databases using the BLAST (Basic Local Alignment Search Tool) similarity search program (available on the internet at www.ncbi.nlm.nih.gov, July 1998), and were therefore deemed "false-positives".

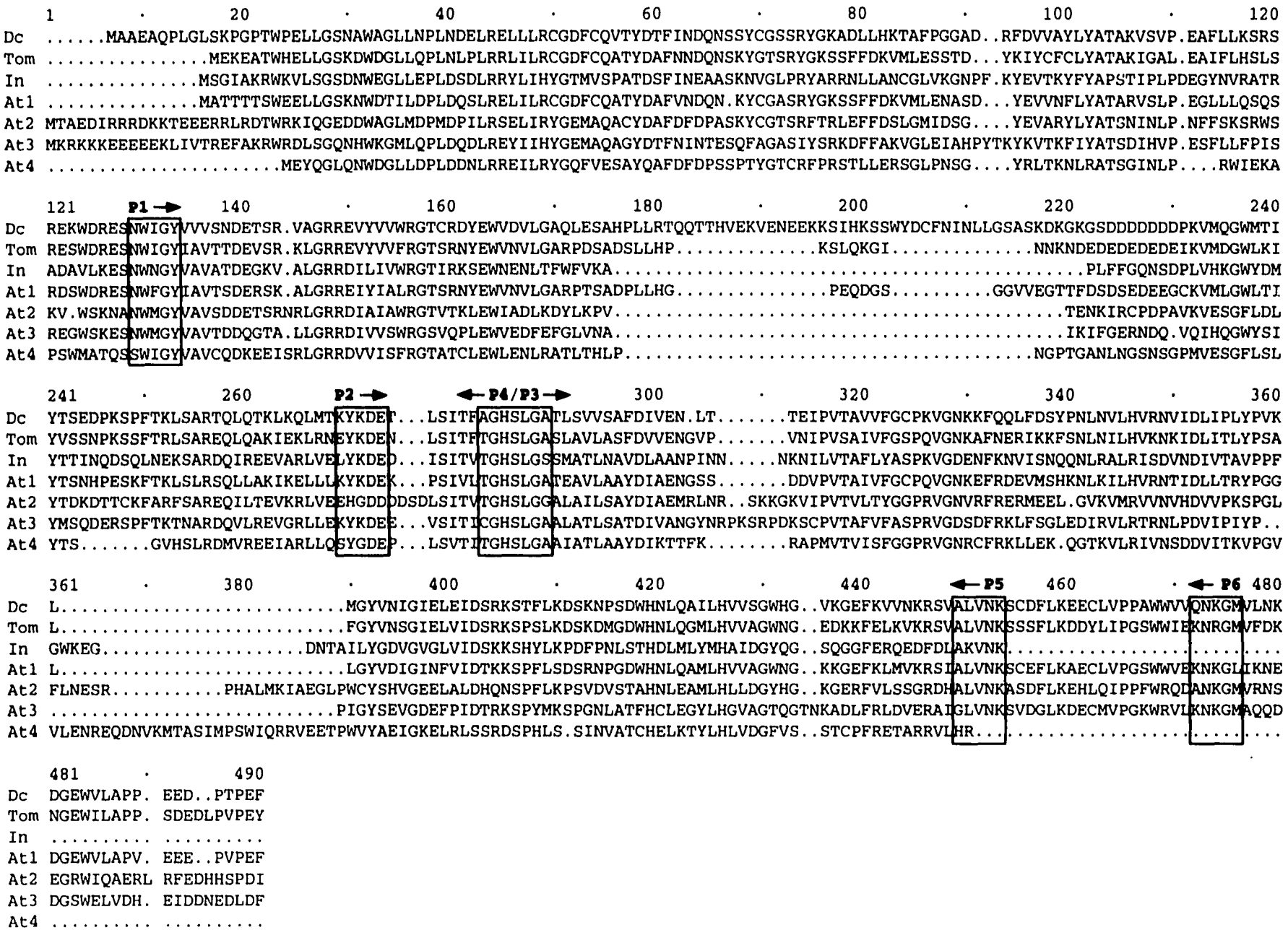
#### *4.2.4.2 Reverse-Transcription Polymerase Chain Reaction*

##### *4.2.4.2.1 Degenerate Primer Design*

As an alternative, RT-PCR was used in an attempt to isolate a partial cDNA corresponding to a lipase gene from the leaves of *Phaseolus vulgaris*. The amino acid sequences deduced from seven different lipase clones from various plant species, including the clone from carnation petals and a lipase clone isolated from tomato fruit (Wang and Thompson, unpublished data), were aligned using MultAlin version 5.3.3 (Corpet, 1988), a multiple sequence alignment program available on the internet (W3.toulouse.inra.fr) (Fig. 4.2). The alignment of the seven lipases allowed for the identification of regions of homology between the sequences. Five regions of high homology, including the lipase consensus sequence pentapeptide (GHSLG), were identified as good candidates for designing corresponding degenerate primers for use in RT-PCR. The degenerate primers were designed using a number of parameters in an attempt to reduce the degeneracy as much as possible. These parameters included: 1. All possible nucleotide sequences (codons) of each clone as deduced from the most common amino acid residues in each region of homology; 2. the actual nucleotide sequence of each clone in the areas of high homology; 3. the "rules" of the wobble hypothesis (e.g. G can form base pairs with C or T); and 4. codon usage of *Phaseolus vulgaris* (using codon usage tables, available on the internet at www.kazusa.or.jp/codon/, June 1999). In addition, special emphasis was placed on the nucleotide sequence of the carnation clone, already known to be up-regulated during senescence, when deciding which nucleotides to include in the degenerate primers.

Three degenerate upstream primers [P1, 5′ cgg caa gct taa (t/c)tg gat (t/c/a)gg (t/g)ta 3′, containing the restriction enzyme site for *Hind*III (underlined); P2, 5′ gcc gtc tag aaa (a/g)ta

Figure 4.2. Aligned amino acid sequences of lipases from various species. The alignment was performed to identify regions of homology between lipases that could be used to design degenerate oligonucleotide primers for RT-PCR amplification of a lipase from *Phaseolus vulgaris* leaves. The five regions of homology that were selected and that correspond to the locations of the six primers that were designed are indicated. P1, location of upstream primer #1; P2, location of upstream primer #2; P3, location of upstream primer #3; P4, location of downstream primer #4; P5, location of downstream primer #5; P6, location of downstream primer #6; Dc, *Dianthus caryophyllus* lipase (Accession No. AAD01804); Tom, tomato lipase (Wang and Thompson, unpublished sequence, personal communication); In, *Ipomea nil* lipase-like protein (Accession No. AAB07724); At1, *Arabidopsis thaliana* putative lipase (Accession No. AAD21737); At2, *Arabidopsis* lipase-like protein (Accession No. CAA16735); At3, *Arabidopsis* lipase isolog (Accession No. AAB63082); At4, *Arabidopsis* triacylglycerol lipase isolog (Accession No. AAC31843). The arrows indicate the direction of extension from each primer. The numbers do not correspond to any one sequence, but are for reference only. The alignment was performed by MultAlin version 5.3.3 (Corpet, 1988), a multiple sequence alignment program available on the internet (W3.toulouse.inra.fr).

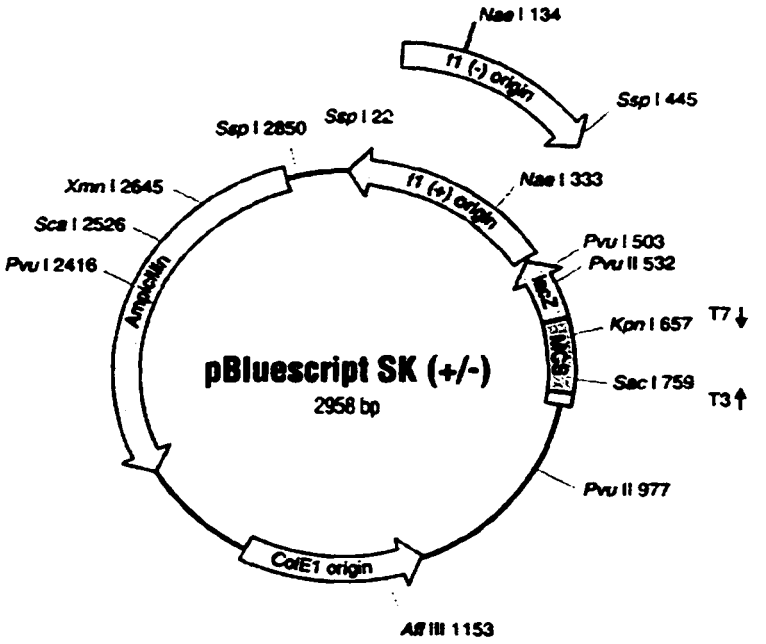

P1
P2
P4/P3
P5
P6
Dc
Tom
In
At1
At2
At3
At4

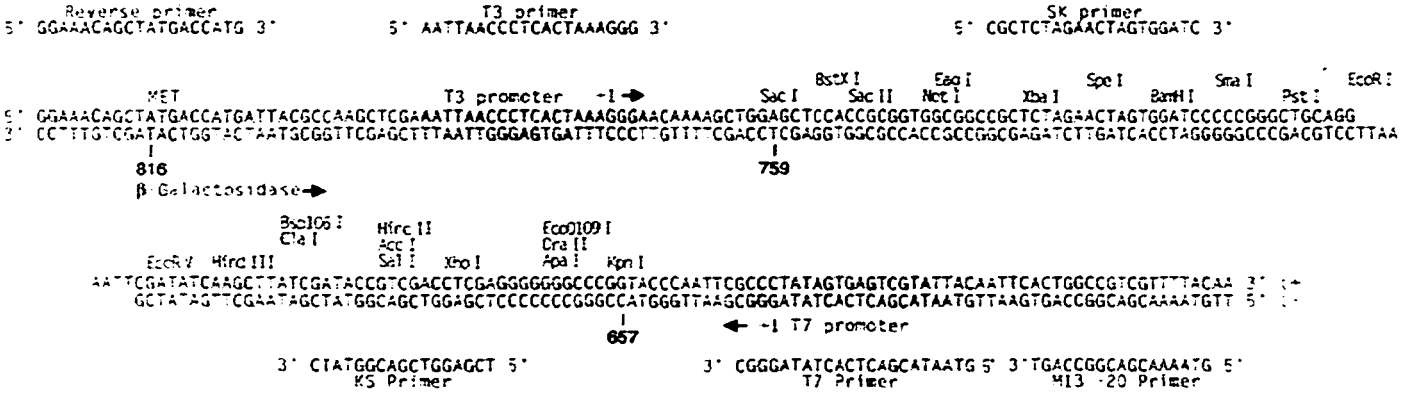
(c/t)aa (a/g)ga (c/t)ga 3′, containing the restriction enzyme site for *Xba*I (underlined); and P3, 5′ gat tgg atc cgc (g/a/c)gg (g/t)ca tag ttt (g/t/a)gg 3′, containing the restriction enzyme site for *BamH*I (underlined)] and three degenerate downstream primers [P4, 5′ aat ctc gag (g/t)(g/c)t gcc (c/a/t)ag gct gtg (g/t)cc 3′, containing the restriction enzyme site for *Xho*I (underlined); P5, 5′ cga cgg tac ctt tgt t(a/g/c)a c(a/g/c)a (a/g)(g/t)g c 3′, containing the restriction enzyme site for *Kpn*I (underlined); and P6, 5′ cga cgg tac cca t(g/t)c c(t/c)t t(a/g)t t(t/c)t 3′, containing the restriction enzyme site for *Kpn*I (underlined)] were ultimately designed and synthesized. All primers had restriction enzyme site linkers as well as 3 or 4 extra nucleotides incorporated into their 5′ ends to facilitate generation of cohesive ends by restriction enzyme digestion for sub-cloning of any RT-PCR products into the multiple cloning site (MCS) of the pBluescript KS (pBS) cloning vector (Fig. 4.3). The primers were synthesized by The Institute for Molecular Biology and Biotechnology (MOBIX, McMaster University, Hamilton, ON). The positions of all six primers and the direction of extension by DNA polymerase from each are indicated on the aligned lipase amino acid sequences shown in Figure 4.2.

#### *4.2.4.2.2 Reverse-Transcription PCR*

RT-PCR was performed using *Ready-To-Go* RT-PCR Beads (Amersham-Pharmacia Biotech). Moloney Murine Leukemia Virus (M-MuLV) reverse transcriptase, *Taq* DNA polymerase, buffer components (10 mM Tris-HCl (pH 9.0), 60 mM KCl, 1.5 mM $MgCl_2$) and 200 μM of each dNTP were all contained within the *Ready-To-Go* beads. The beads were dissolved in DEPC-treated water as per the supplier's instructions (Protocol 3) to give a final reaction volume of 50 μL. First-strand cDNA synthesis was accomplished by the addition of 5 μg of total RNA isolated from the primary leaves of 3-week-old *Phaseolus vulgaris* seedlings

Figure 4.3. Diagram of pBluescript KS (Stratagene) cloning vector used to sub-clone RT-PCR products obtained from *Phaseolus vulgaris* leaf RNA, amplified with degenerate lipase primers. The multi-cloning site, located adjacent to the *lacZ* gene, is shown in detail.


Nae I 134
f1 (-) origin
Ssp I 445
Sap I 2850
Sap I 22
Xmn I 2645
f1 (+) origin
Nae I 333
Sca I 2526
Pvu I 503
Pvu I 2416
Pvu II 532
lacZ
Ampicillin
Kpn I 657
T7
pBluescript SK (+/-)
2958 bp
Sac I 759
T3
Pvu II 977
ColE1 origin
Afl III 1153


Reverse primer
5' GGAAACAGCTATGACCATG 3'
T3 primer
5' AATTAACCCTCACTAAAGGG 3'
SK primer
5' CGCTCTAGAACTAGTGGATC 3'
BstX I
Eag I
Spe I
Sma I
EcoR I
MET
T3 promoter
-1
Sac I
Sac II
Not I
Xba I
BamH I
Pst I
5' GGAAACAGCTATGACCATGATTACGCCAAGCTCGAAATTAACCCTCACTAAAGGGAACAAAAGCTGGAGCTCCACCGCGGTGGCGGCCGCTCTAGAACTAGTGGATCCCCCGGGCTGCAGG
3' CCTTTGTCGATACTGGTACTAATGCGGTTCGAGCTTTAATTGGGAGTGATTTCCCTTGTTTTCGACCTCGAGGTGGCGCCACCGCCGGCGAGATCTTGATCACCTAGGGGGCCCGACGTCCTTAA
816
759
β-Galactosidase
Bsc106 I
Cla I
Hinc II
Acc I
Sal I
Xho I
Eco0109 I
Cra II
Apa I
Kpn I
EcoR V
Hinc III
AATTCGATATCAAGCTTATCGATACCGTCGACCTCGAGGGGGGGCCCGGTACCCAATTCGCCCTATAGTGAGTCGTATTACAATTCACTGGCCGTCGTTTTACAA 3'
GCTATAGTTCGAATAGCTATGGCAGCTGGAGCTCCCCCCCGGGCCATGGGTTAAGCGGGATATCACTCAGCATAATGTTAAGTGACCGGCAGCAAAATGTT 5'
657
-1 T7 promoter
3' CTATGGCAGCTGGAGCT 5'
KS Primer
3' CGGGATATCACTCAGCATAATG 5'
T7 Primer
3' TGACCGGCAGCAAAATG 5'
M13 -20 Primer

that was first denatured by incubation for 5 min at 65°C, as the template, and 2.5 μg of a random hexamer ($pd(N)_6$, Amersham-Pharmacia Biotech) as the primer for M-MuLV reverse transcriptase. Synthesis of cDNA was achieved by incubation at 42°C for 30 min in a thermal-cycler (Perkin Elmer, GeneAmp PCR System 2400), followed by a 5 min incubation at 95°C to inactivate the reverse transcriptase. Degenerate primers were then added to the mixture for amplification of any cDNAs corresponding to a lipase, by PCR. Upstream primers (P1, P2, and P3) and downstream primers (P4, P5, and P6) were added (100 pmol each) separately in the following pairs: P1-P4, P1-P5, P1-P6, P2-P4, P2-P5, P2-P6, P3-P5, P3-P6. Using the programmable thermal cycler, amplification was performed for 35 cycles. Each cycle consisted of denaturation at 95°C for 1 min, annealing at 48°C for 1 min, and extension at 72°C for 2 min, with a final extension at 72°C for 15 min. Following amplification by PCR, the DNA mixtures were separated using agarose gel electrophoresis. Table 4.1 lists the predicted sizes for the RT-PCR products produced from each primer pair based on the amino acid sequence of the carnation lipase clone.

#### *4.2.4.3 Sub-Cloning of RT-PCR Products*

RT-PCR products of the predicted size were excised from the agarose gel and eluted using the GeneClean II purification kit according to the instructions (BIO 101). An RT-PCR product amplified by primer pair P1-P4 was double-digested with *Hind*III/*Xho*I; a product amplified by primer pair P1-P5 was digested with *Hind*III/*Kpn*I; a product amplified by primer pair P3-P5 was digested with *BamH*I/*Kpn*I; and a product amplified by primer pair P3-P6 was digested with *BamH*I/*Kpn*I. All four restriction enzyme-digested RT-PCR products were then sub-cloned into the multiple cloning site of pBS vectors that had been digested with the same pairs

Table 4.1. Predicted sizes[a] of RT-PCR products and corresponding amino acid peptides obtained using degenerate lipase primers for amplification of partial lipase cDNA clones from *Phaseolus vulgaris* leaves.

| Primer Pair | Predicted Size | |
|---|---|---|
| | RT-PCR Product (bp) | Corresponding Peptide (# A.A.) |
| P1-P4 | 471 | 157 |
| P1-P5 | 849 | 283 |
| P1-P6 | 918 | 306 |
| P2-P4 | 378 | 126 |
| P2-P5 | 756 | 252 |
| P2-P6 | 825 | 275 |
| P3-P5 | 399 | 133 |
| P3-P6 | 468 | 156 |

[a]The predictions are based on the lipase cDNA clone isolated from carnation petals (see Fig. 4.1)

of restriction enzymes. To ligate the double-digested PCR product with the appropriately double-digested pBS, the two were mixed in a ratio of 3:1 (PCR product : plasmid on a per weight basis). Ligation was achieved by addition of 1 Unit of T4 DNA ligase, and incubation in ligation buffer (60 mM Tris-HCl, 5 mM $MgCl_2$, 1 mM dithioerythritol, and 1 mM ATP, pH 7.5) overnight at 15°C, according to the supplier's instructions (Boehringer Mannheim/Roche).

#### *4.2.4.4 Transformation and Selection*

The next day, the ligation mixture was mixed with 100 µL of competent *E. coli* DH5-α cells (made competent using RbCl/CaCl method; Kushner, 1978), and placed on ice for 30 min. The cells were then transferred to 42°C for 90 sec to facilitate uptake of the plasmid DNA by the competent cells. After the 90 sec incubation, 200 µL of Luria-Bertani (LB) broth (containing [per litre] 10 g NaCl, 5 g yeast extract, and 10 g tryptone, adjusted to pH 7.0 with NaOH) was added, and the cells were further incubated at 37°C for 45 min. During the 45 min incubation, 16 µL of 50 mg/ml X-Gal and 8 µL of 100 mM IPTG were added to LB agar plates containing 100 µg/mL ampicillin. After the 45 min incubation, aliquots of transformed cells were spread onto the prepared plates. The plates were then incubated at 37°C overnight.

Only transformants can grow on the ampicillin-containing medium as pBS provides ampicillin resistance to the cells. Transformants were selected based on the blue-white selection scheme which distinguishes between cells transformed by recombinant pBS (white colonies) and re-circularized pBS (blue colonies). IPTG induces the expression of the *lacZ* gene of pBluescript, which contains the multi-cloning site, by de-repression. Specifically, IPTG binds to the repressor protein produced by the *lacI* gene, preventing it from binding and therefore inactivating the *lacZ* promoter. In plasmids that have re-circularized without

incorporating a PCR product, functional *lacZ* gene product (β-galactosidase) will be produced. β-galactosidase is capable of converting the substrate X-Gal (provided in the growth medium) into a blue product. Therefore, any bacterial colonies that appear blue contain re-circularized pBS with no insert. However, in plasmids that have ligated with a PCR product and therefore have incorporated this DNA into their MCS, expression of the *lacZ* gene is disrupted. In this case, the gene no longer produces functional β-galactosidase, even when induced by IPTG. Therefore, cells that contain recombinant pBS and therefore non-functional *lacZ* genes are incapable of converting the X-Gal into a blue product and appear white. It is these white colonies that contain recombinant plasmids of interest. Five white colonies were randomly selected from each of four plates. Each plate represented one of the four RT-PCR products that had been selected for further analysis on the basis of their size.

### 4.2.5 Sequencing of RT-PCR Products

The recombinant plasmids from the selected transformed colonies were purified from overnight cultures of each, using the Wizard Plus DNA Purification kit (Promega). Sequencing of the RT-PCR inserts was performed by MOBIX (McMaster University, Hamilton, ON) using the T3 and T7 primers, which have complementary binding sites upstream and downstream of the MCS of pBS, respectively. MOBIX uses a fluorescence-based automated DNA sequencing approach, a reaction cycle of 96°C (melting), 50°C (annealing) and 60°C (extension), and *Taq* FS polymerase for extension.

The nucleotide sequence of each insert was converted to the corresponding amino acid sequence using the "Translate Tool" available on the ExPASy Molecular Biology Server on the internet (www.expasy.ch) in all six possible reading frames (three forward frames and

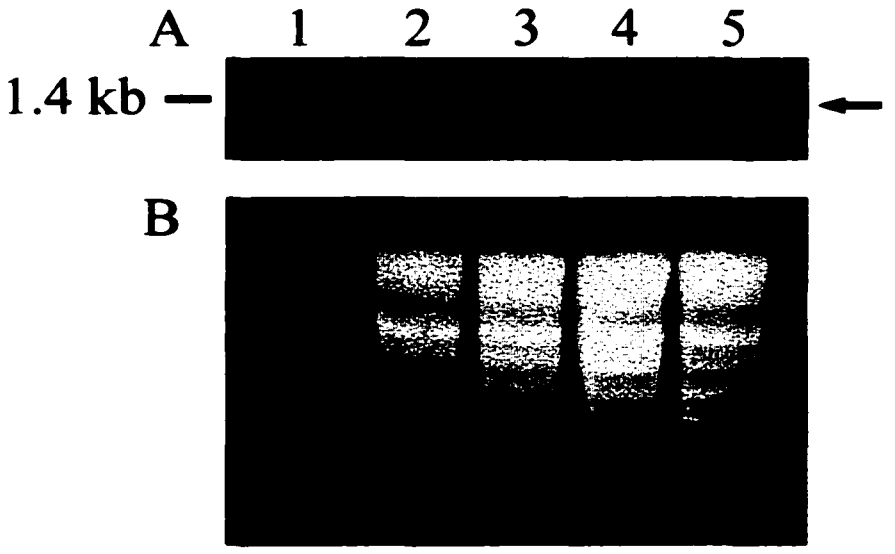
three reverse frames). The resulting deduced amino acid sequences were observed for any potential open reading frames. Three potential open reading frames were identified and subsequently compared to the protein data banks using the BLAST search program, available on the National Center for Biotechnology Information (NCBI) internet site (www.ncbi.nlm.nih.gov/, June 1999). As well, the sequences of the carnation lipase and three partial *Phaseolus* clones were analyzed for putative phosphorylation sites using PhosphoBase v2.0, a phosphorylation site prediction program available on the internet (www.cbs.dtu.dk/, November 1999). Finally, the amino acid sequences of some full-length lipases available from protein databanks that shared high degrees of sequence similarity to the three partial leaf lipase clones, were analyzed for potential chloroplast-targeting signals using ChloroP V1.1, a program for predicting chloroplast-targeted proteins based on their amino acid sequences that is available on the internet (http:\\genome.cbs.dtu.dk, September 1999).

## 4.3 RESULTS

### 4.3.1 Identification of a Lipase that is Up-regulated during Senescence of *Phaseolus vulgaris* Primary Leaves

Total RNA extracted from *Phaseolus vulgaris* primary leaves of different ages was probed on a Northern blot using a heterologous lipase cDNA probe from carnation petals. As shown in Figure 4.4, a transcript from *Phaseolus vulgaris* leaves that is of a similar size to the carnation lipase mRNA (kindly provided by Michael Wang) is detected by the carnation lipase probe. The transcript is barely detectable in young tissue (1-week-old leaves) and increases in abundance with advancing age of the primary leaves to show a strong signal for 3-week-old tissue (Fig. 4.4, lanes 2, 3 and 5). In addition, it appears that expression of this

Figure 4.4. Northern blot of total RNA isolated from the primary leaves of *Phaseolus vulgaris* probed with a heterologous carnation lipase cDNA clone showing increased expression of a leaf lipase during senescence. A, RNA blot probed with radiolabeled carnation lipase clone. Lane 1, total carnation petal RNA (5 μg); lane 2, total RNA isolated from 1-week-old *Phaseolus vulgaris* primary leaves (10 μg); lane 3, total RNA isolated from 2-week-old *Phaseolus vulgaris* primary leaves (10 μg); lane 4, total RNA isolated from 2-week-old *Phaseolus vulgaris* primary leaves after treatment with 1 ppm ethylene for 16 hours (10 μg); lane 5, total RNA isolated from 3-week-old *Phaseolus vulgaris* primary leaves (10 μg). Arrow indicates position of band. B, Ethidium bromide-stained agarose gel as it appeared before transfer of the RNA to the nylon membrane.


A
1
2
3
4
5
1.4 kb
B

transcript is up-regulated following treatment of 2-week seedlings with exogenous ethylene, a senescence-accelerating hormone (Fig. 4.4, lanes 3 and 4).

The total protein and chlorophyll content was also measured for leaves of the ages probed for expression of lipase mRNA. Table 4.2 shows that both protein and chlorophyll content of the leaves (measured on a per fresh weight basis) declined as a function of time. The amount of chlorophyll per gram fresh weight in 3-week leaves was slightly less than that of the younger leaves. However, the amount of protein per gram fresh weight declined steadily as a function of time and was considerably lower in 3-week leaves than any of the younger leaves (Table 4.2). It has been demonstrated previously that the leaves of 3-week-old seedlings are at the early stages of senescence (Roberts et al., 1987).

### 4.3.2 Isolation and Sequencing of Lipase cDNA Clones Expressed in *Phaseolus vulgaris* Leaves

An attempt was made to isolate a lipase cDNA clone from a cDNA library constructed from the mRNA of mature (~2-week-old) *Phaseolus vulgaris* primary leaves. The library was screened with a heterologous lipase cDNA probe isolated from carnation petal cytosol. This yielded only "false positives", and no clones that corresponded to a lipase. It was reasoned that this was due to the fact that the library was made using mRNA from relatively young tissue, and that consequently any lipases corresponding to the senescence-induced carnation lipase clone used to probe the leaf library might not be abundant enough to detect using the heterologous probe.

As an alternative to screening the cDNA library, an RT-PCR approach was used in an attempt to isolate a partial lipase clone expressed in *Phaseolus vulgaris* leaves. Six degenerate lipase primers were designed and used in all possible combinations to amplify any lipase

Table 4.2. Chlorophyll and protein content of leaves used for isolation of total RNA for Northern blot analysis

| Leaf Age (weeks) | Chlorophyll[b] (mg·g fresh wt.$^{-1}$) | Protein[b] (mg·g fresh wt.$^{-1}$) |
|---|---|---|
| 1 | 0.23 | 8.5 |
| 2 | 0.25 | 6.3 |
| 2 + $C_2H_4$[a] | 0.23 | 6.3 |
| 3 | 0.19 | 2.8 |

[a]2-week-old seedlings treated for 16 h with 1 ppm ethylene in a sealed chamber before analysis
[b]Results from one experiment representative of three separate experiments are shown

sequences by PCR. mRNA from 3-week-old primary leaves was used as a template for PCR amplification, and a random hexamer was used as the primer for reverse transcriptase. Primer pair P1-P4 produced a ~450 bp PCR product; primer pair P1-P5 produced a ~500 bp PCR product; primer pair P3-P5 produced a ~400 bp PCR product; and primer pair P3-P6 produced a ~450 bp PCR product. All four PCR products were of similar size to that predicted on the basis of the carnation lipase amino acid sequence (Fig. 4.2, and Table 4.1). RT-PCR amplification using the other primer pairs (P1-P6, P2-P4, P2-P5, P2-P6) either resulted in PCR products of an unpredicted size, or no products at all.

The RT-PCR products of the predicted size produced by the four primer pairs (P1-P4, P1-P5, P3-P5 and P3-P6) were sequenced and the corresponding amino acid sequences determined. All of the RT-PCR products obtained using the P1-P4 degenerate primer pair had the same deduced 134 amino acid sequence (Fig. 4.5). Degenerate primer pair P3-P6, however, produced two different open reading frames. The first ORF produced by primer pair P3-P6 was designated P3-P6a and corresponded to a 160 amino acid peptide (Fig. 4.6). The second ORF produced by primer pair P3-P6 was designated P3-P6b and corresponded to a 157 amino acid peptide (Fig. 4.7). The RT-PCR products obtained using the other two primer pairs (P1-P5 and P3-P5) did not correspond to open reading frames when translated in any of the six potential reading frames.

#### *4.3.2.1 Sequence Analysis of Putative* **Phaseolus vulgaris** *Leaf Lipases*

A BLAST search (September 1999) revealed that the 134 amino acid sequence deduced from the P1-P4 RT-PCR product (Fig. 4.5) shared 50% identity and 65% similarity with a putative

Figure 4.5. Amino acid sequence of partial lipase cDNA clone obtained by RT-PCR from *Phaseolus vulgaris* leaves using degenerate lipase primers P1 and P4. The partial clone has been aligned with the corresponding partial sequences of five other lipases commonly returned by BLAST searches using this and other partial leaf lipases obtained by RT-PCR. The alignment was performed by MultAlin version 5.3.3 (Corpet, 1988), a multiple sequence alignment program available on the internet (W3.toulouse.inra.fr). Only those residues that are shared by the partial *Phaseolus vulgaris* leaf lipase and at least one other aligned sequence are highlighted. P1-P4, amino acid sequence of a partial *P. vulgaris* leaf lipase cDNA obtained by RT-PCR using degenerate lipase primers P1 and P4; Dc, *Dianthus caryophyllus* lipase (Accession No. AAD01804); In, *Ipomea nil* lipase-like protein (Accession No. AAB07724); At1, *Arabidopsis thaliana* putative lipase (Accession No. AAD21737); At2, *Arabidopsis* lipase-like protein (Accession No. CAA16735); At3, *Arabidopsis* lipase isolog (Accession No. AAB63082); At5, Arabidopsis putative lipase (Accession No. AAC63840).

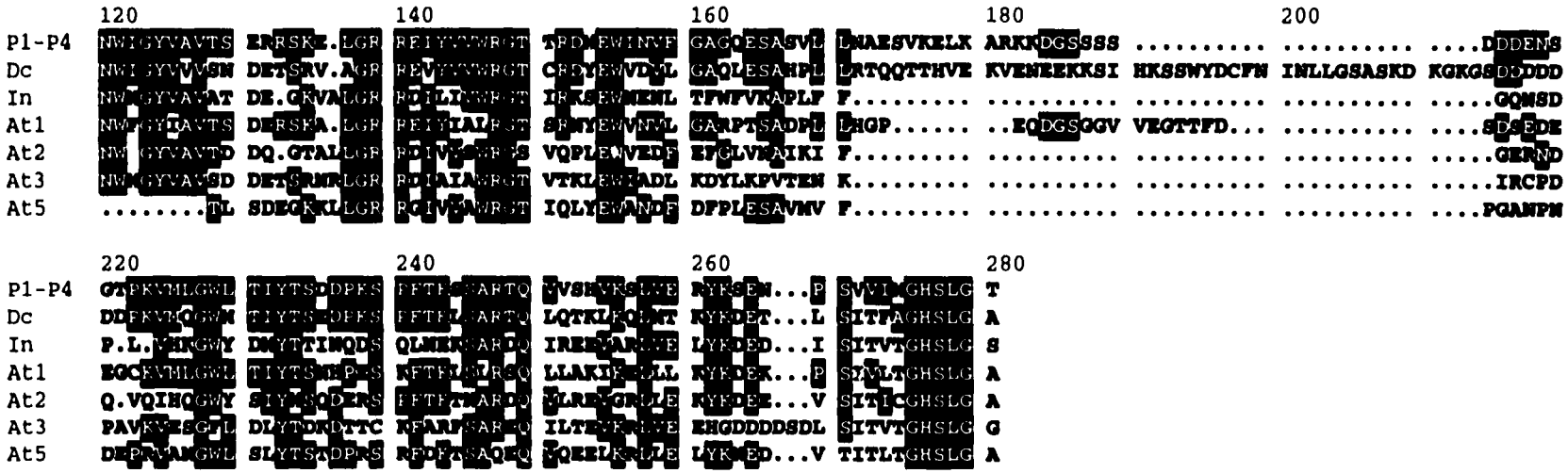

120
140
160
180
200
P1-P4
Dc
In
At1
At2
At3
At5
220
240
260
280
P1-P4
Dc
In
At1
At2
At3
At5

Figure 4.6. Amino acid sequence of partial lipase cDNA clone obtained by RT-PCR from *Phaseolus vulgaris* leaves using degenerate lipase primers P3 and P6. The partial clone has been aligned with the corresponding partial sequences of five other lipases commonly returned by BLAST searches using this and other partial leaf lipases obtained by RT-PCR. The alignment was performed by MultAlin version 5.3.3 (Corpet, 1988), a multiple sequence alignment program available on the internet (W3.toulouse.inra.fr). Only those residues that are shared by the partial *Phaseolus vulgaris* leaf lipase and at least one other aligned sequence are highlighted. P3-P6a, amino acid sequence of a partial *P. vulgaris* leaf lipase cDNA obtained by RT-PCR using degenerate lipase primers P3 and P6; Dc, *Dianthus caryophyllus* lipase (Accession No. AAD01804); In, *Ipomea nil* lipase-like protein (Accession No. AAB07724); At1, *Arabidopsis thaliana* putative lipase (Accession No. AAD21737); At2, *Arabidopsis* lipase-like protein (Accession No. CAA16735); At3, *Arabidopsis* lipase isolog (Accession No. AAB63082); At5, Arabidopsis putative lipase (Accession No. AAC63840).

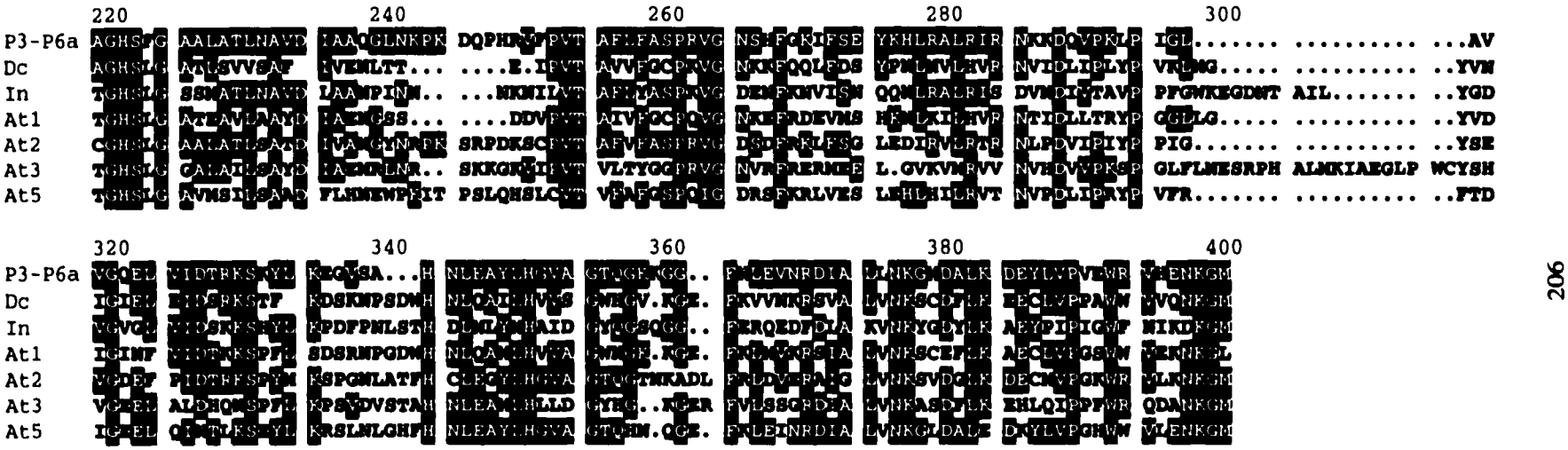
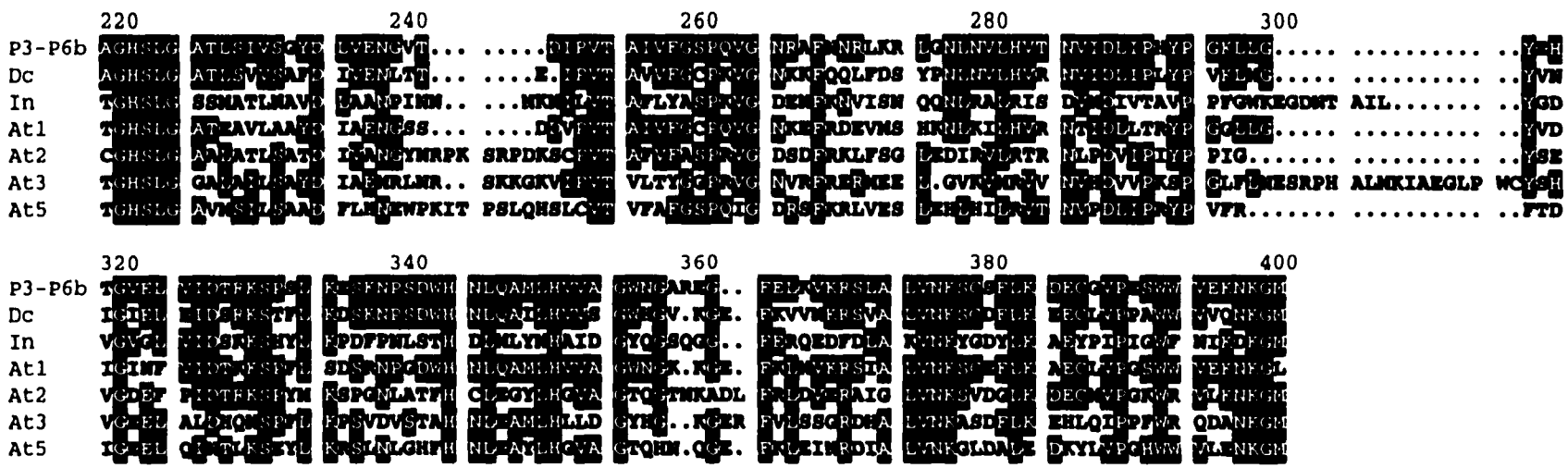

220
240
260
280
300
320
340
360
380
400
P3-P6a
Dc
In
At1
At2
At3
At5

Figure 4.7. Amino acid sequence of partial lipase cDNA clone obtained by RT-PCR from *Phaseolus vulgaris* leaves using degenerate lipase primers P3 and P6. The partial clone has been aligned with the corresponding partial sequences of five other lipases commonly returned by BLAST searches using this and other partial leaf lipases obtained by RT-PCR. The alignment was performed by MultAlin version 5.3.3 (Corpet, 1988), a multiple sequence alignment program available on the internet (W3.toulouse.inra.fr). Only those residues that are shared by the partial *Phaseolus vulgaris* leaf lipase and at least one other aligned sequence are highlighted. P3-P6b, amino acid sequence of a partial *P. vulgaris* leaf lipase cDNA obtained by RT-PCR using degenerate lipase primers P3 and P6; Dc, *Dianthus caryophyllus* lipase (Accession No. AAD01804); In, *Ipomea nil* lipase-like protein (Accession No. AAB07724); At1, *Arabidopsis thaliana* putative lipase (Accession No. AAD21737); At2, *Arabidopsis* lipase-like protein (Accession No. CAA16735); At3, *Arabidopsis* lipase isolog (Accession No. AAB63082); At5, Arabidopsis putative lipase (Accession No. AAC63840).


220
240
260
280
300
P3-P6b
Dc
In
At1
At2
At3
At5
320
340
360
380
400
P3-P6b
Dc
In
At1
At2
At3
At5

lipase protein from *Arabidopsis thaliana* (Accession No. AC006931), with an E value of 5 x $10^{-31}$ (Table 4.3). No gaps were introduced into either sequence in order to properly align them. As well, the other eight "hits" returned by the BLAST search corresponded to either lipase or "lipase-like" enzymes. Included in this list of similar sequences was the senescence lipase from carnation petal cytosol that was used to probe the Northern blot in Figure 4.4 (Accession No. AAD01804). The sequence of the partial P1-P4 clone from *Phaseolus* shared 43% identity and 57% similarity with the lipase from carnation, with an E value of 4 x $10^{-30}$ (Table 4.3). Figure 4.5 shows the partial P1-P4 *P. vulgaris* leaf lipase clone aligned with the amino acid sequences of six other lipases. The six other lipases with which the alignment was performed were selected on the basis of their appearance in the list of BLAST hits for this partial lipase clone, as well as the other two partial lipase clones obtained (see below). Only that portion of the sequences that corresponds to the partial *P. vulgaris* lipase sequence was included in the alignment, and only those residues that are shared by the *P. vulgaris* sequence and at least one other lipase sequence are highlighted (Fig. 4.5).

The deduced amino acid sequence from the first of the partial clones obtained from the degenerate primer pair P3-P6 (designated P3-P6a; Fig. 4.6), shared 57% identity and 68% similarity with a lipase-like protein from *Arabidopsis thaliana* (Accession No. AL021710) as determined by a BLAST similarity search (Table 4.4; September 1999). This "hit" had an E value of 4 x $10^{-42}$, and included gaps accounting for 3% of the sequence (Table 4.4). The same putative lipase from *Arabidopsis* (Accession No. AC006931) that was 50% identical to the partial P1-P4 *Phaseolus* lipase clone was also included in the list of 32 "hits" for this clone, as was the lipase from carnation petal cytosol. Figure 4.6 shows the amino acid sequence of the partial P3-P6a clone aligned with the corresponding region of six other lipases. Only those

Table 4.3. Top five hits returned by a BLAST similarity search (September 1999) for the partial cDNA clone obtained by RT-PCR using degenerate lipase primers P1 and P4 and total RNA isolated from 3-week-old *Phaseolus* primary leaves as template.

| Clone Name | Accession Number | Species Name | % Identity | % Positive | E-Value | % Gaps |
|---|---|---|---|---|---|---|
| Putative Lipase | AAD21737 | *Arabidopsis thaliana* | 50 | 65 | $5 \times 10^{-31}$ | 0 |
| Lipase | AAD01804 | *Dianthus caryophyllus* | 43 | 57 | $4 \times 10^{-30}$ | 15 |
| Lipase-like Protein | CAA16735 | *Arabidopsis thaliana* | 40 | 56 | $9 \times 10^{-21}$ | 15 |
| Putative Lipase | AAC63840 | *Arabidopsis thaliana* | 37 | 53 | $3 \times 10^{-16}$ | 15 |
| Lipase | AAB07724 | *Ipomea nil* | 34 | 44 | $4 \times 10^{-14}$ | 16 |

Table 4.4. Top five hits returned by a BLAST similarity search (September 1999) for the first partial cDNA clone (P3-P6a) obtained by RT-PCR using degenerate lipase primers P3 and P6 and total RNA isolated from 3-week-old *Phaseolus* primary leaves as template.

| Clone Name | Accession Number | Species Name | % Identity | % Positive | E-Value | % Gaps |
|---|---|---|---|---|---|---|
| Lipase-like Protein | CAA16735 | *Arabidopsis thaliana* | 57 | 68 | $4 \times 10^{-42}$ | 3 |
| Putative Lipase | AAC63840 | *Arabidopsis thaliana* | 47 | 62 | $2 \times 10^{-35}$ | 2 |
| Lipase | AAB07724 | *Ipomea nil* | 42 | 59 | $7 \times 10^{-28}$ | 11 |
| Lipase | AAD01804 | *Dianthus caryophyllus* | 42 | 56 | $1 \times 10^{-27}$ | 8 |
| Putative Lipase | AAD21737 | *Arabidopsis thaliana* | 43 | 55 | $2 \times 10^{-26}$ | 7 |

residues that are shared by the *P. vulgaris* sequence and at least one other lipase sequence are highlighted (Fig. 4.6).

Included in the 49 BLAST hits (Table 4.5; September 1999) found for the amino acid sequence of the second partial clone obtained from the degenerate primer pair P3-P6 (designated P3-P6b; Fig. 4.7), were the putative lipase (Accession No. AC006931) and lipase-like protein (Accession No. AL021710) from *Arabidopsis* that were identified as close matches to the other two partial clones. However, the hit with the highest identity to the P3-P6b partial clone was identified as the lipase from carnation petal cytosol. This 157 amino acid peptide shared 66% identity and 80% similarity with the carnation lipase clone. There were no gaps in the aligned sequences and the E value for the hit was $2 \times 10^{-57}$ (Table 4.5). The alignment of this partial lipase amino acid sequence with six other lipases is shown in Figure 4.7.

Analysis of the carnation lipase clone and three partial *Phaseolus* clones for putative phosphorylation sites was performed using PhosphoBase v2.0. The analysis revealed a number of potential sites at which all four polypeptides could be phosphorylated (Figure 4.8). Phosphorylation of these proteins at the motifs identified by the prediction program (or others not identified by the program) could be involved in the regulation of the enzymes.

## 4.4 DISCUSSION

*Phaseolus vulgaris* RNA probed using a heterologous carnation lipase cDNA clone on a Northern blot demonstrated that a lipase is up-regulated in primary leaves that are in the early stages of senescence, specifically 21 days after planting. Chlorophyll and protein measurements confirmed that the leaves in which the lipase is most strongly expressed are at

Table 4.5. Top five hits returned by a BLAST similarity search (September 1999) for the second partial cDNA clone (P3-P6b) obtained by RT-PCR using degenerate lipase primers P3 and P6 and total RNA isolated from 3-week-old *Phaseolus* primary leaves as template.

| Clone Name | Accession Number | Species Name | % Identity | % Positive | E-Value | % Gaps |
|---|---|---|---|---|---|---|
| Lipase | AAD01804 | *Dianthus caryophyllus* | 66 | 80 | $2 \times 10^{-57}$ | 0 |
| Putative Lipase | AAD21737 | *Arabidopsis thaliana* | 64 | 76 | $5 \times 10^{-56}$ | 0 |
| Putative Lipase | AAC63840 | *Arabidopsis thaliana* | 45 | 59 | $7 \times 10^{-30}$ | 6 |
| Lipase-like Protein | CAA16735 | *Arabidopsis thaliana* | 46 | 56 | $7 \times 10^{-30}$ | 7 |
| Lipase Isolog | AAB63082 | *Arabidopsis thaliana* | 40 | 54 | $2 \times 10^{-28}$ | 13 |

Figure 4.8. **Predicted phosphorylation sites for A, carnation petal lipase (Accession No. AAD01804); B, putative bean leaf lipase P1-P4; C, putative bean leaf lipase P3-P6a; and D, putative bean leaf lipase P3-P6b. The predictions were made by PhosphoBase v2.0, a phosphorylation site prediction program available on the internet (Kreegipuu et al., 1999; www.cbs.dtu.dk/databases/PhosphoBase/), based on consensus sequence motifs. Potential phosphorylation sites for various protein kinases, including CaMII, CKI, CKII, PKA, PKC and PKG, are highlighted in black. The lipase consensus pentapeptide is underlined for reference.**


A
MAAEAQPLGLSKPGPTWPELLGSMAWAGLLNPLNDELRELLLRCGDFCQVTYDTFIMDQNSSYCGSSRYGKADLLH
KTAFPGGADRFDVVAYLYATAKVSVPEAFLLKSRSREKWDRESNWIGYVVVSNDETSRVAGRREVYVVWFGTCRDY
EWVDVLGAQLESAHPLLRTQQTTHVEKVENEEKKSIHKSSWYDCFNIMLLGSASKDKGKGSDDDDDDPKVMQGWM
TIYTSEDPKSPFTKLSARTQLQTKLKQLMTKYKDETLSITFAGHSLGATLSVVSAFDIVENLTTEIPVTAVVFGCP
KVGNKKFQQLFDSYPNLNVLHVRNVIDLIPLYPVKLMGYVNIGIELEIDSPKSTFLKDSKNPSDWHNLQAILHVVS
GWHGVKGEFKVVNKRSVALVNKSCDFLKEECLVPPAMWVVQNKGMVLMKDGEWVLAPPEEDPTPEFD
B
NWIGYVAVTSERRSKELGRREIYVVWRGTTRDMEWINVFGAGQESASVLLNAESVKELKARKKDGSSSSDDDENSG
TPKVMLGWLTIYTSDDPKSPFTKSSARTQVVSHVKSLVERYKSENPSVVIMGHSLGT
C
AGHSFGAALATLNAVDIAAQGLNKPKDQPHRVFPVTAFLFASPRVGNSHFGKIFSEYKHLRALRIRNKKDQVPKLP
IGLAVVGQELVIDTRKSKYLKEGVSAHNLEAYLHGVAGTQGKKGGFNLEVNRDIALLNKGMDALKDEYLVPVEWRV
HENKGM
D
AGHSLGATLSIVSGYDLVENGVTDIPVTAIVFGSPQVGNRAFNNRLKRLGNLNVLHVTNVIDLIPHYPGKLLGYEH
TGVELVIDTRKSPSLKESKNPSDWHNLQAMLHVVAGWNGAREGFELKVKRSLALVNKSCSFLKDECGVPESWWVEK
NKGMG

the early stages of senescence. Indeed, it has been shown previously that *Phaseolus* primary leaves begin to senesce approximately 3 weeks after planting (Tevini, 1976; Fong and Heath, 1977; Bate et al., 1991). It has also been demonstrated that *Phaseolus* leaves contain active galactolipid-degrading activity (Sastry and Kates, 1964; Helmsing, 1969; Anderson et al., 1974; Matsuda et al., 1979; Burns et al., 1980), and that the membrane lipid content of leaves declines dramatically during senescence (Tevini, 1976; Fong and Heath, 1979). Moreover, it has been reported that the galactolipid content of leaves declines more rapidly than the phospholipid content (Draper, 1969; Ferguson and Simon, 1973; Dalgarn et al., 1979). Therefore, it seems logical that there is an enzyme that specifically degrades galactolipids in leaf chloroplasts. However, while it has long been assumed that a galactolipase must be involved in chloroplast membrane senescence (Galliard, 1980), a direct relationship between a galactolipase and the senescence-specific breakdown of galactolipids has never been conclusively proven. Indeed, the only physiological roles so far proposed for lipase capable of degrading galactolipids based on direct supporting evidence is in the chilling sensitivity of some species (Kaniuga et al., 1999), and drought tolerance in others (Sahsah et al., 1998).

It has also been proposed that chloroplast lipid metabolism plays a significant role in the regulation of leaf senescence (Gepstein, 1988). This stems in particular from the finding that hydrolysis of the thylakoid galactolipids in a non-yellowing mutant of *Festuca pratensis* was impaired in comparison to the wild type (Harwood et al., 1982). Degradation of thylakoid membranes and their pigments during senescence is also impaired in this mutant. It has been proposed that altered lipid metabolism in the *Festuca* mutant (i.e. limited galactolipid degradation) slows the degradation of the thylakoid in general (Harwood et al., 1982).

It has also been proposed that galactolipases are involved in turnover of chloroplast membrane lipids (Burns et al., 1980; Douce and Joyard, 1980). Galactolipids are known to be turned over rapidly (Appelqvist, 1975; Woolhouse, 1984). This turnover requires an active and specific lipolytic enzyme, and such activity has been isolated from leaves and chloroplasts (Sastry and Kates, 1964; Helmsing, 1969; Anderson et al., 1974; Matsuda et al., 1979; Burns et al., 1980). However, as with the putative role of galactolipases in senescence, no direct link has been made between galactolipid turnover and galactolipase activity in mature leaves. In fact, relatively little is known about the catabolic aspects of lipid turnover in plants (Galliard, 1980; Woolhouse, 1984).

The finding that a lipase is up-regulated during the early stages of senescence in bean leaves, and in response to exogenous ethylene, prompted an effort to isolate the gene(s) involved. To this end, a cDNA library made from the mRNA of mature primary bean leaves was screened with the heterologous senescence-induced carnation lipase cDNA clone in an effort to isolate the corresponding gene from leaves. However, this analysis yielded only "false-positives", and was abandoned. The failure to isolate a corresponding clone from *Phaseolus* leaves in this way with the carnation clone might have been expected, as the carnation clone is up-regulated during senescence, and the cDNA library was constructed from the mRNA of non-senescing leaves. Accordingly, an RT-PCR approach was utilized in an attempt to isolate a lipase gene from bean leaves.

Degenerate primers were designed based on the amino acid and nucleotide sequences of seven plant lipases. These primers were used in all possible combinations to amplify putative lipase sequences from the cDNA generated from RNA isolated from 3-week-old primary leaves. A random hexamer was used as primer for reverse transcriptase. This analysis resulted

in the isolation of three partial open reading frames (named P1-P4, P3-P6a and P3-P6b), all of which contained the lipase consensus sequence pentapeptide. Indeed, when compared to the protein databanks by BLAST search analysis, only plant lipases were found to be significantly similar to the three partial *Phaseolus* sequences. Therefore, it appears that these three partial sequences correspond to lipases that are expressed in the primary leaves of 3-week-old *Phaseolus vulgaris* seedlings.

The full-length clones of the three lipases have not yet been isolated, and therefore the precise substrate specificity (i.e. identification of the exact class of lipase to which they belong) and their subcellular localization have not yet been determined. However, it is possible to speculate on the identity and localization of the enzymes encoded by the three clones. For instance, it is possible that one of the three partial clones represents the lipase that was observed to be up-regulated at the onset of senescence, by the Northern blot probed with the heterologous carnation lipase clone. Indeed, clone P3-P6b is very similar to the carnation lipase clone at the amino acid level (66% identical, 80% positive), as determined by a BLAST similarity search. This level of similarity and the low E-value suggest that clone P3-P6b may represent the corresponding senescence-induced lipolytic acyl hydrolase from *Phaseolus* leaves.

There are other possible identities for the three putative leaf lipases. Indeed, any of the three lipases may correspond to lipolytic acyl hydrolases with different specificities, including galactolipases similar to those from *Phaseolus* leaves that have been characterized biochemically (Sastry and Kates, 1964; Helmsing, 1969; Anderson et al., 1974; Matsuda et al., 1979; Burns et al., 1980). Indeed, galactolipids are known to be rapidly turned over in actively photosynthesizing leaves and also decline rapidly, more rapidly than phospholipids, during

senescence (Draper, 1969; Tevini, 1976; Woolhouse, 1984). As well, it has been reported that galactolipid-degrading activity of *Phaseolus* leaves is high (Galliard, 1980), and although such activity has been isolated from leaves, a gene for such an enzyme has not yet been described.

In support of speculation that one of these clones could be a galactolipase, there is evidence that at least one of the putative lipases may be localized to the chloroplast. Specifically, clone P3-P6a is 50% identical and 57% similar to a putative TAG lipase from *Arabidopsis* (Accession No. AAD24845) as determined by BLAST comparison of the two sequences (September 30, 1999). This putative *Arabidopsis* lipase is predicted to contain a cleavable N-terminal chloroplast targeting sequence of 63 amino acids, using ChloroP V1.1, a program for predicting chloroplast-targeted proteins based on their amino acid sequences. This program is available on the internet (http:\\genome.cbs.dtu.dk, September 1999). As chloroplasts are very rich in galactolipids, accounting for ~80% of the polar lipids (Douce and Joyard, 1980), it is logical to speculate that a lipase targeted to the chloroplast is very likely a galactolipase. Therefore, the identification of the *Arabidopsis* clone (Accession No. AAD24845) as a putative TAG lipase may be incorrect, and partial *Phaseolus* clone P3-P6a may be targeted to the chloroplast where it degrades galactolipids. None of the three lipases likely correspond to phospholipases, as the BLAST searches did not find similarity to any such enzymes (see Tables 3, 4, and 5). However, the lipases could be neutral lipid-degrading lipases (true TAG lipases) as many of the lipases returned by the BLAST searches are thought to degrade triacylglycerols, and contain the TAG lipase semi-conserved ten-amino-acid sequence. Indeed, evidence does exist that TAG and other neutral esters are found in leaves and chloroplasts (Harwood, 1980; Harwood et al., 1982; Steinmüller and Tevini, 1985). It has

also been reported that TAG accumulates in plastids in response to stress (Sakaki et al., 1990). It therefore makes sense that a neutral lipid-degrading lipase would be targeted to chloroplasts.

The full length clones for each of the three partial *Phaseolus* lipases remain to be isolated. Once isolated, analysis of the sequences will show if clone P1-P4 is the upstream portion of either of P3-P6a or P3-P6b, or if the three partial clones represent portions of three separate lipase isoforms. It remains possible that one of P3-P6a and P3-P6b are the downstream portion of the P1-P4 clone. A PCR approach was utilized in an attempt to determine if this was the case, but was unsuccessful. Specific primers were designed based on the nucleotide sequences of the three partial clones. That is, an upstream primer was designed based on the nucleotide sequence of clone P1-P4, and two downstream primers were designed based on the nucleotide sequences of P3-P6a and P3-P6b clones. These three specific primers were used in combination to amplify a portion of any potential corresponding lipases from *Phaseolus vulgaris* genomic DNA. This did not result in the amplification of any PCR products. It is possible that these results indicate that the three partial lipase clones represent three distinct lipase isoforms. However, it cannot yet be ruled out that one of P3-P6a and P3-P6b represents the downstream portion of P1-P4.

Isolation of the full-length genes will also allow for characterization of the cognate enzymes, and also allow for the use of these proteins to generate polyclonal antibodies. The antibodies will be necessary to determine the sub-cellular localization and temporal expression patterns of the lipases. Full-length clones will be isolated by generating a cDNA library from the mRNA of 3-week-old leaves and screening this library as well as an existing cDNA library made from the mRNA of 2-week-old primary leaves with the three partial clones. Screening of these two libraries should allow for the isolation of lipase(s) expressed in young/mature

tissue, as well as lipases expressed in leaf tissue at the onset of senescence (3-week-old primary bean leaves). Presumably, lipases expressed in young/mature leaves would be involved in turnover and/or stress responses, whereas those enzymes expressed at later stages of leaf development would be involved in senescence-associated lipid breakdown.

It is also possible to speculate on the possible mode(s) of regulation of the putative leaf lipases. To this end, the carnation petal lipase and putative leaf lipases have been analyzed for putative phosphorylation motifs using the PhosphoBase v2.0 phosphorylation site prediction program (Kreegipuu et al., 1999), which is available on the internet (www.cbs.dtu.dk/, November 1999). PhosphoBase predicts multiple potential phosphorylation sites in all 4 sequences analyzed (Fig. 4.8). It is worth noting that some of these motifs are shared by more than one of the lipases examined. For example, motifs TIYTSED and TKLSART of the carnation lipase (Fig. 4.8A) are very similar to motifs TIYTSDD and TKSSART of bean leaf lipase clone P1-P4 (Fig. 4.8B). Furthermore, motif SRKSTFLK of the carnation lipase (Fig. 4.8A) is very similar to motifs TRKSKYLK and TRKSPSLK of bean leaf lipases P3-P6a and P#-P6b, respectively (Figs. 4.8, C and D). It is possible that some of these sites, or sites not identified by the program, could be phosphorylated/dephosphorylated to regulate the activity of the lipases. Indeed, it has been proposed that a lipase from potato leaves is regulated by phosphorylation/dephosphorylation (Moreau, 1986) Furthermore, it is possible that calcium and calmodulin regulate some of the leaf lipases. Indeed, it has been suggested that calcium and calmodulin may be directly or indirectly involved in regulating lipolytic acyl hydrolase activity responsible for degrading leaf polar lipids (Lesham et al., 1984; Moreau and Isett, 1985). On the other hand, it has also been suggested that $Ca^{2+}$ does not stimulate lipolytic acyl hydrolases that are involved in membrane deterioration (Paliyath and Droillard, 1992).

However, the possibility that calcium and/or calmodulin might be involved is worth investigating.

Finally, the association of lipases with lipid bodies from other tissues (Lin et al., 1983; Hoppe and Theimer, 1997), the similarity of chloroplastic lipid-protein particles/plastoglobuli to oil bodies and cytosolic lipid-protein particles and the high free fatty acid content of chloroplastic lipid bodies collectively indicate that a lipase (probably a galactolipase) may be associated with the chloroplastic lipid bodies. While this cannot yet be confirmed, generation of a leaf lipase antibody will aid in determining if such a lipase is indeed associated with the chloroplastic particles or thylakoid membranes.

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# 5. DISCUSSION

Lipid bodies termed lipoproteins from mammals and other animals are very well characterized (Murphy and Vance, 1999). Considerably less information is available, however, about lipid bodies from plants. To date, three broad classes of plant lipid bodies have been identified: storage oil bodies from seeds, cytoplasmic lipid-protein particles involved in membrane turnover and organellar lipid bodies such as those found in plastids.

Oil bodies are the best characterized types of plant lipid bodies. Their general structure is fully elucidated. However, there is still uncertainty surrounding their complete protein composition, their exact mechanism of formation and their metabolism (Matsui et al., 1999). Cytosolic lipid-protein particles were first described in 1991 for cotyledons of etiolated *Phaseolus vulgaris* seedlings, and have since been identified in a variety of plant tissues (Yao et al., 1991; Thompson et al., 1998). Lipid-protein particles resemble oil bodies in that they also contain a neutral lipid core that includes triacylglycerol and appear to be surrounded by a monolayer of phospholipids (Hudak and Thompson, 1996). However, lipid-protein particles contain less triacylglycerol than oil bodies and are enriched in free fatty acids and protein metabolites. It has, therefore, been proposed that cytosolic lipid-protein particles are involved in membrane turnover rather than lipid storage (Thompson et al., 1998). Moreover, it is unclear whether cytosolic lipid-protein particles contain a structural protein analogous to oleosin.

Plastid lipid bodies, termed plastoglobuli, were first described for spinach leaf chloroplasts in 1961 and have subsequently been observed in all types of plastids in numerous species (Park and Pon, 1961; Lichtenthaler, 1968). However, details of their exact chemical composition,

structure and function remain elusive. For example, although their neutral lipid content has been largely elucidated, only recently was it shown that they do in fact contain galactolipids, and are most likely delineated by a monolayer of these polar lipids (Hernández-Pinzón et al., 1999). Recent studies have also indicated that plastoglobuli contain numerous proteins, some of which belong to a family of lipid-associated proteins referred to as plastid-lipid associated proteins (PAPs), fibrillins or plastoglobulins (Pozueta-Romero et al., 1997; Kessler et al., 1999). Because plastoglobuli are abundant in developing chloroplasts before thylakoid biogenesis, and increase in size and abundance in parallel with thylakoid dissolution during senescence, it is generally believed that these lipid bodies serve as reservoirs for thylakoid constituents. Although the possibility that they may also have other physiological functions has not been ruled out (Lichtenthaler, 1968), little, if any, work has been done to determine what those other functions might be.

Another class of chloroplastic lipid bodies, termed lipid-protein particles, has recently been isolated from the stroma of *Phaseolus vulgaris* leaf chloroplasts (Ghosh et al., 1994). These lipid-protein particles are analogous to those isolated from cytosol, and although they are obtained from chloroplasts, they differ from plastoglobuli in that they are not isolated by flotation centrifugation. As well, plastoglobuli are isolated from sonicated chloroplasts. Sonication serves to release plastoglobuli from between thylakoid lamellae. Lipid-protein particles, on the other hand, are isolated from the stroma of chloroplasts not subjected to sonication. Stromal lipid-protein particles contain neutral esters, free fatty acids, diacylglycerol and galactolipids, as well as a number of thylakoid proteins and their catabolites (Ghosh et al., 1994). It has, therefore, been suggested that these chloroplastic lipid-protein particles are involved in thylakoid turnover, in much the same way as their cytosolic

counterparts appear to be involved in turnover of membranes exposed to the cytosol, such as the plasma membrane (Thompson et al., 1998). Specifically, chloroplastic lipid-protein particles are thought to provide a mechanism for removal of membrane catabolites such as free fatty acids, diacylglycerol and protein catabolites, that would otherwise destabilize the structure of the thylakoid if allowed to accumulate (Ghosh et al., 1994). Although stromal lipid-protein particles bear morphological resemblance to plastoglobuli, differences in size, composition and the procedures used for their isolation prompted recent speculation that they may have different functions (Thompson et al., 1998). As well, plastoglobuli are generally abundant in young, developing chloroplasts and senescing chloroplasts, whereas, stromal lipid-protein particles are isolated from mature, actively photosynthesizing leaves.

The objective of the current study was to characterize more fully the chemical composition of chloroplastic lipid-protein particles, and in so doing define more precisely their role in thylakoid membrane turnover. Furthermore, it was expected that determination of their chemical composition might also reveal other functions for the lipid-protein particles in chloroplasts. The final goal was to isolate a lipase clone from leaves that might be involved in thylakoid membrane metabolism by encoding an enzyme responsible for the high levels of free fatty acids detected in lipid-protein particles.

Stromal lipid-protein particles have previously been shown to contain galactolipids as well as some thylakoid proteins and their catabolites (Ghosh et al., 1994). Thylakoid proteins that have been detected include the $CF_1$ $\beta$ and $\gamma$ subunits of ATPase, cytochrome *f* and the 33-kD and D2 subunits of PSII (Ghosh et al., 1994). These observations lead to the conclusion that lipid-protein particles originate from thylakoids. In the present study, the demonstration that the fatty acid compositions of lipid-protein particles and thylakoids are very similar supports

this contention. Indeed, the lipid-protein particles contain the same fatty acids as thylakoids, albeit in different relative proportions. Specifically, the lipid-protein particles contain relatively less linolenic acid than thylakoids, but are still enriched in this fatty acid as compared to cytosolic lipid-protein particles from carnation petals and etiolated *Phaseolus vulgaris* cotyledons (McKegney et al., 1995; Hudak and Thompson, 1996). The lower concentration of this fatty acid in lipid-protein particles relative to thylakoids may reflect selective inclusion of certain membrane components into the particles, or may indicate that particles are formed from specific sites along the plane of the membrane. In addition, the finding that lipid-protein particles with very similar properties to those isolated from the stroma can be generated *in vitro* from light-incubated thylakoids is further evidence that the particles do indeed originate from the membranes. Furthermore, the *in vitro* formation of lipid-protein particles from isolated thylakoids is almost completely eliminated by prior heat denaturation, and incubation of thylakoids in light generates approximately 2-fold more particles than incubation in darkness. Finally, the addition of ATP to the dark-incubated thylakoids partially alleviates the inhibitory effect of darkness on the formation of particles. Collectively, these observations indicate that stromal lipid-protein particles do originate from thylakoids, and that their formation is physiologically regulated. The *in vitro* light-generated lipid-protein particles were examined by transmission electron microscopy, and their size was determined by dynamic light-scattering. This demonstrated that the *in vitro*-generated particles are of similar size and morphology to the stromal lipid-protein particles.

Indications that the formation of stromal lipid-protein particles is an integral aspect of thylakoid turnover together with the morphological similarities they share with plastoglobuli and oil bodies prompted an examination of whether these lipid particles contain a structural

protein analogous to oleosin of oil bodies. A plastid-lipid associated protein, which is thought to be a structural component of plastoglobuli, has recently been described (Pozueta-Romero et al., 1997). Indeed, various proteins determined to be members of this PAP/fibrillin family based on amino acid sequence similarities have now been identified as integral components of plastoglobuli in various types of plastids from numerous species (Kessler et al., 1999; Hernández-Pinzón et al., 1999). The notion that the proteins of this family are structural components of lipid particles is supported by hydropathy analyses, which reveal hydrophobic domains capable of penetrating into a lipid matrix (Vishnevetsky et al., 1996; Ting et al., 1998; Chapter 3). In addition, members of the PAP/fibrillin family have been localized to the exterior surface of plastoglobuli, and in this sense resemble oleosin, which is present on the outer surface of oil bodies (Pozueta-Romero et al., 1997; Kessler et al., 1999).

To examine the relationship between plastoglobuli and stromal lipid-protein particles and to determine whether the lipid-protein particles share with plastoglobuli the property of possessing PAP, both were isolated from the chloroplasts of mature *Phaseolus vulgaris* primary leaves. Plastoglobuli were isolated from sonicated chloroplasts by flotation centrifugation, and lipid-protein particles were isolated by ultrafiltration of the stroma from non-sonicated chloroplasts following high-speed centrifugation. Western blots probed with antibody raised against PAP of *Capsicum annuum* revealed that both plastoglobuli and lipid-protein particles are enriched in the protein as compared to thylakoids. The finding that lipid-protein particles contain a significant amount of PAP suggests that these lipid bodies are in fact plastoglobuli-like particles distinguishable from floated plastoglobuli on the basis of differences in buoyant density. In addition, the supernatant that formed beneath the floated pad of plastoglobuli was collected and also found to contain considerable amounts of PAP.

This finding confirms that there are multiple sub-populations of plastoglobuli-like particles in chloroplasts that can be distinguished based on their buoyant densities. Presumably the differences in buoyant densities reflect differences in protein-to-lipid ratios of the different lipid bodies, which may in turn reflect functional differences. Formation of membrane metabolites is accelerated during times of stress, such as drought, and it is conceivable that plastoglobuli and/or lipid-protein particles are also involved in the re-modeling of thylakoids under these circumstances. The relationship between the two particle types requires further examination, but it is possible that they are formed from different sites along the plane of the membrane, thus explaining differences in their chemical compositions. Perhaps, for example, plastoglobuli are formed preferentially from granal lamellae, accounting for their close association with thylakoids. Conversely, it seems reasonable to propose that lipid-protein particles are formed from stromal lamellae, and can thus be easily isolated from the stroma without sonication.

One of the characteristic features of stromal lipid-protein particles is that they contain certain thylakoid proteins as well as proteolytic fragments of these proteins (Ghosh et al., 1994). Considering the finding that lipid-protein particles resemble plastoglobuli in that they contain PAP, the possibility that plastoglobuli might also contain thylakoid protein catabolites was examined. To this end, both lipid-protein particles and plastoglobuli were probed on Western blots for the presence of cytochrome *f*, a thylakoid protein that had been previously identified as a constituent of lipid-protein particles (Ghosh et al., 1994). This examination revealed that lipid-protein particles contain at least two lower molecular weight forms of cytochrome *f* that were shown to be cytochrome *f* catabolites. Additionally, Western blotting revealed that plastoglobuli contain the most abundant of the lower molecular weight

cytochrome *f* catabolites and that the higher-density plastoglobuli-like particles collected from the supernatant beneath the floated pad of plastoglobuli contained the same catabolites as the stromal lipid-protein particles. These compositional similarities may reflect functional similarities among the various particle types. In particular, the results suggest a possible role for plastoglobuli and lipid-protein particles in normal thylakoid turnover, and specifically in the removal of damaged proteins and their catabolites from the membrane. Together with the finding that plastoglobuli and lipid-protein particles also both contain PAP, it seems likely that these two particle types are generically related. However, the differences in fatty acid and overall protein compositions indicate that they may also have distinct functions in addition to the functions that they appear to share.

Cytochrome *f* was used as a marker protein to more closely examine the lipid-protein particles and their role in thylakoid turnover. Antibodies raised against synthetic peptides corresponding to the termini of cytochrome *f* were used to determine what portions of the protein are associated with the lipid-protein particles. Probing of Western blots with these antibodies indicated that all the lower molecular weight forms of cytochrome *f* associated with the particles and thylakoids lack portions of both termini. As well, co-elution of cytochrome *f* catabolites with PAP and fatty acids from a Sephacryl size-exclusion column indicated that the proteins are genuine components of a lipid complex. Furthermore, comparison of the catabolites with a recombinant, truncated version of cytochrome *f* indicates that the largest, most abundant catabolite contains the hydrophobic α-helix of the protein. Presumably, this α-helix is embedded in a lipid matrix, as it is in thylakoids.

The finding that the largest of the cytochrome *f* catabolites is associated with thylakoids in a lower relative abundance than with the particles is consistent with the suggestion that this

catabolite is formed on the membrane and released into the stroma in the form of a lipid-protein particle. The association of various forms of cytochrome *f* with the particles, including what appears to be full-length protein in some instances, indicates that any damaged or non-functional form of the protein can be voided from the membrane by the formation of lipid-protein particles. Alternatively, it is possible that the multiple forms of the protein represent cytochrome *f* at various stages of degradation, and that this step-wise degradation occurs after release from thylakoids. It has been suggested that a cytochrome *f*-specific protease may be associated with thylakoids of some species (Gray, 1992). However, this protease is thought to have a specific site of cleavage and may not be capable of generating all forms of the protein detected in the present study. Therefore, it is possible that damaged proteins are initially cleaved at the level of the thylakoid and subsequently degraded after their release from the membrane in association with lipid-protein particles.

In the present study, it has been demonstrated that a sub-population of stromal lipid-protein particles can be isolated by flotation centrifugation. Interestingly, it was found that this sub-population of particles shares with *in vitro*-generated particles and washed thylakoid membranes the property of possessing the large subunit of Rubisco (RLSU), but no detectable Rubisco small subunit (RSSU). Indeed, this observation suggests that the particles may be involved in more than simply thylakoid membrane turnover, and this possibility was examined more closely.

It has been reported that free, precursor RLSU and, in some cases, Rubisco holoenzyme can associate with thylakoids (Kirk, 1978; Mori et al., 1984; Makino and Osmond, 1991). In addition, RLSU can be translated on thylakoid-bound polysomes (Bhaya and Jagendorf, 1984; Hattori and Margulies, 1986), which are responsible for co-translational insertion of proteins

into thylakoids, and it is synthesized with an N-terminus that is cleaved before incorporation into the holoenzyme (Langridge, 1981; Amiri et al., 1984). It is conceivable that this N-terminal extension could act as a thylakoid-targeting signal, allowing association of RLSU with the membrane. However, a physiological function for unassembled large subunit associated with thylakoids has not been proposed. It has, however, been suggested that free, precursor RLSU is associated with thylakoids prior to its incorporation into Rubisco holoenzyme, although no specific details have been suggested (Hattori and Margulies, 1986; Klein et al., 1988; Makino and Osmond, 1991). The possibility that RLSU can associate with lipids is in agreement with the well-known hydrophobic character of the polypeptide (Gatenby, 1984). Furthermore, it has been suggested that the inability to induce the assembly of Rubisco holoenzyme *in vitro* arises from the difficulty of forming the soluble $L_8$ core of the enzyme (a known assembly intermediate) from the individual, insoluble large subunits (Gutteridge and Gatenby, 1995; Roy and Gilson, 1997). Therefore, it seems possible that large subunit could associate with thylakoids prior to being incorporated into $L_8S_8$ Rubisco and that a membrane- or lipid-associated step could be involved in holoenzyme assembly as proposed by Makino and Osmond (1991).

In view of these previous reports, the finding in the present study that RLSU is associated with thylakoids and enriched in lipid-protein particles in the absence of small subunit was examined more closely. A number of observations confirm that the large subunit found associated with lipid-protein particles is a genuine, integral component and not simply non-specifically adsorbed to the surface of the particles. These observations include the behavior of particle-associated large subunits in a Sephacryl size-exclusion column, as compared to individual large subunits dissociated from holoenzyme by treatment with 8 M urea. Lipid-

protein particle-associated RLSU elutes from the column in much earlier fractions than individual RLSU liberated from the holoenzyme, indicating that the particle-associated polypeptide is part of a larger complex. Evidence that this complex contains lipid includes the finding that a spectrum of fatty acids similar to those detected in stromal lipid-protein particles co-precipitates with RLSU following immunoprecipitation of stromal lipid-protein particles with Rubisco antibody. Furthermore, the fatty acids of the immuoprecipitated lipid-protein particles are present in a similar free-to-esterified fatty acid ratio as is found in particles before immunoprecipitation. In addition, proteinase K shaving experiments generate a protease-protected fragment of particle-associated large subunit that is also detectable in thylakoids, but there is no protease-protected fragment when isolated Rubisco holoenzyme is treated with the protease. Lipid constituents of the particles presumably protect this portion of the protein from proteolysis. Finally, incubation of particle-free stroma containing Rubisco with cytosolic lipid-protein particles and microsomal membranes isolated from etiolated bean cotyledons indicates that the association of RLSU with chloroplastic particles and membranes is not non-specific in nature. This is due to the finding that Rubisco did not non-specifically adhere to the cytosolic particles. The small amount of RLSU that did associate with microsomal membranes was not converted to a smaller protected fragment by proteinase K as was the case for thylakoids, but rather was completely degraded in a short period of time. Therefore, it seems reasonable to suggest that RLSU associated with thylakoids and lipid-protein particles is genuine and has a physiological function.

Collectively, these observations are consistent with the contention that lipid-protein particles bearing RLSU originate from thylakoids and that particle-associated RLSU is destined to be assembled into Rubisco holoenzyme in the stroma. Specifically, the lipid-

protein particles may act as a platform for the formation of the $L_8$ core of Rubisco during holoenzyme assembly. It is also possible that the large subunit found embedded in lipid-protein particles represents misfolded polypeptide that is destined for degradation rather than assembly. However, this seems unlikely given that Western blots show that cpn60, which is involved in Rubisco $L_8S_8$ assembly, is also associated with stromal lipid-protein particles isolated by flotation.

Interestingly, a recent report describes chloroplast lipid particles that appear to be secreted from chloroplasts into the cytoplasm, where they are degraded (Guiamét et al., 1999). These lipid bodies, referred to as "blebs", differ from chloroplastic lipid-protein particles and plastoglobuli in that they contain chlorophyll. However, like lipid-protein particles, they also contain thylakoid proteins. It is possible that secretion of chloroplast lipid particles into the cytoplasm is necessary for the degradation of some chloroplast components. Perhaps a subset of chloroplast lipid bodies is secreted for degradation, while others are degraded in the stroma. Indeed, it has been demonstrated in the present study that multiple subpopulations of lipid bodies exist within the chloroplasts of *Phaseolus vulgaris* leaves. These subpopulations can be distinguished based on differences in density. Specifically, higher density lipid-protein particles can be isolated from the stroma by ultrafiltration of the supernatant following high-speed centrifugation. High-density plastoglobuli have also been isolated by high-speed centrifugation of sonicated chloroplasts, and lower-density plastoglobuli have been isolated by flotation centrifugation of sonicated chloroplasts. While these particles differ in their buoyant densities, they all share the property of possessing PAP. Together with the finding that they also all contain catabolites of the photosynthetic thylakoid protein, cytochrome *f*, it would

appear that the different classes of lipid particles have common functions. Indeed, plastoglobuli and stromal lipid-protein particles may both be involved in thylakoid turnover.

The mechanism of formation of chloroplast lipid particles has yet to be elucidated, but it is possible that PAP, or other as yet unidentified proteins, are involved (Pozueta-Romero et al., 1997). Alternatively, formation of lipid particles may be a purely biophysical process, initiated by the lateral phase separation of membrane metabolites forming domains that are voided from the thylakoid as particles (Sackman and Feder, 1995). However, it seems most likely that the mechanism of formation actually involves some combination of both of these alternatives, and may be similar to the mechanism proposed for oil body genesis from the endoplasmic reticulum (Murphy and Vance, 1999).

It has been proposed that proteins must be voided from membranes, including thylakoids, in association with lipids, such that fatty acids act as detergents for the hydrophobic protein domains (Thomas and Hilditch, 1987). However, the mechanism by which these proteins are removed from membranes has not been definitively elucidated. The contention that it involves lipid gained support when it was demonstrated that photosynthetic proteins could be isolated from the stroma in association with lipid-protein particles (Ghosh et al., 1994). Using cytochrome *f* as a marker protein, this hypothesis has been more strongly substantiated in the present study. It seems clear that damaged versions of at least some of the proteins of the thylakoid membrane are voided in association with lipids, presumably during the process of normal thylakoid turnover. The proteins and lipids of the lipid-protein particles are likely further degraded after their release. It is possible that this degradation occurs in the stroma, although there is also evidence to suggest that particles are secreted to the cytoplasm for degradation (Guiamét et al., 1999). Indeed, the details of the downstream metabolism of

chloroplastic lipid particles once they have been removed from thylakoids remain unknown. It seems logical that, once removed from the membrane, the lipids, proteins and metabolites that make up lipid-protein particles and plastoglobuli must be degraded to complete the catabolic side of normal turnover. The details of this degradation are not at all clear. However, it has been proposed that PAP may be involved in targeting lipid bodies for further metabolism (Kessler et al., 1999).

There is some evidence that a triacylglycerol lipase may associate with seed oil bodies (Lin et al., 1983; Hoppe and Theimer, 1997), and other reports indicate that patatin-like phospholipase $A_2$ and lipoxygenase associate with and are involved in the metabolism of cucumber cotyledon oil bodies during germination (May et al., 1998; Matsui et al., 1999). In addition, it has been reported that protease activity is localized in cytosolic lipid-protein particles of etiolated bean cotyledons (Yao and Thompson, 1993). Although there are no reports of degradative enzymes associated with lipid bodies from plastids, it seems reasonable to propose that such enzymes exist.

Northern blot analysis of *Phaseolus* leaf RNA using a heterologous carnation lipase clone as probe revealed that a transcript of the same size as the lipase transcript from carnation is expressed in leaves of 3-wk-old seedlings at the onset of senescence. RT-PCR was used to isolate putative lipase clones from *Phaseolus* leaves that conceivably encode enzymes mediating lipid metabolism in membranes and lipid particles, and may correspond to the transcript identified by Northern blotting. To this end, RNA isolated from 3-week-old primary leaves (at the onset of senescence) was used as template, and six degenerate lipase primers were used in all possible combinations in an attempt to amplify putative lipase sequences from the leaf RNA. This amplification yielded three partial cDNA clones that each corresponded to

plant lipases and putative plant lipases upon comparison to protein databanks using the BLAST search tool. Many of the most similar clones returned by the BLAST similarity searches were predicted to be triacylglycerol (TAG) lipases based on their amino acid sequences. However, the substrate specificities for many of the lipases returned by the search have not been determined experimentally. Therefore, it is possible that these putative TAG lipases may actually be lipolytic acyl hydrolases with varying degrees of substrate specificity, as TAG lipases and lipolytic acyl hydrolases, such as the carnation petal lipase, have amino acid sequence similarities (Fig. 4.2). As such, it is difficult to accurately speculate on the substrate specificity of the three partial leaf lipase clones based on sequence data alone. However, numerous possibilities exist. They may correspond to triacylglycerol lipases in agreement with the prediction based on sequence data, as it has been reported that TAG can accumulate in leaf chloroplasts. Indeed, TAG is an abundant component of plastoglobuli, and it accumulates in chloroplasts in response to stress (Harwood, 1980; Harwood et al., 1982; Steinmuller and Tevini, 1985). Presumably, therefore, there is a need for an enzyme capable of degrading TAG in chloroplasts. The partial leaf lipase clones could also correspond to galactolipases, enzymes that are essential components of chloroplast metabolism and have been shown to be particularly abundant in *Phaseolus* leaves (Galliard, 1980). If one or more of the partial leaf lipases were a galactolipase it would be necessary for these enzymes to be targeted to chloroplasts. The partial sequences of the clones do not allow for prediction of cellular targeting for the corresponding proteins. However, clone P3-P6a is 50% identical to a putative *Arabidopsis* TAG lipase (Accession No. AAD24845) that is predicted to be targeted to the chloroplast by a 63 amino acid signal sequence. It is not unreasonable to predict the same cellular location for clone P3-P6a based on the degree of identity it shares with this

*Arabidopsis* lipase. It is probable that galactolipids would be among the substrates for a lipase targeted to chloroplasts, considering that approximately 80% of the polar lipids in chloroplasts are galactolipids. If in fact one of the three lipases is targeted to chloroplasts and is capable of degrading galactolipids, it would be the first galactolipase to be cloned.

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