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MOLECULAR AND CELLULAR CHARACTERIZATION OF NON-SPECIFIC LIPID TRANSFER PROTEINS FROM SPINACH AND *ARABIDOPSIS*

By

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ABSTRACT

Molecular and cellular characterization of non-specific lipid transfer proteins from spinach and *Arabidopsis*

By

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The mechanism by which lipids move from their main sites of synthesis, the endoplasmic reticulum (ER) and chloroplast, to other cellular organelles, is unknown. Lipid transfer proteins (LTPs) are a class of proteins which are capable of mediating lipid transfer between natural and artificial membranes *in vitro*. Despite a lack of experimental evidence, it has been assumed that LTPs carry out a similar role *in vivo*. This dissertation describes the investigation into the *in vivo* function of plant LTPs. Using *in vitro* transcription and translation in the presence of microsomal membranes, it was shown that a spinach LTP is cotranslationally inserted into the ER. To determine the exact location of LTP in plants, antibodies were raised against an *Arabidopsis* LTP:protein A fusion protein which had been produced in *Escherichia coli*. Using immunoelectron microscopy, the *Arabidopsis* LTP was localized to the cell wall, and was observed predominantly in epidermal cells. To investigate temporal and spatial expression patterns of LTPs, transgenic plants containing an *Arabidopsis* LTP promoter-ß glucuronidase fusion were produced. The LTP promoter was active in very specific cell and tissue types, and

in young seedlings was regulated in a developmental manner. The localization of LTP to the cell wall, and its expression in a specific subset of cell and tissue types precludes any role that this protein may have in lipid transfer. To elucidate the role of LTP within a plant, transgenic *Arabidopsis* containing a greatly reduced level of LTP due to the presence of an *Arabidopsis* cDNA in reverse orientation behind a constitutive promoter, were produced. These plants exhibit no marked phenotype to indicate the function of LTP. The predominance of LTP in epidermal cell walls led to the hypothesis that it may be involved in cuticle formation. However, analysis of wax and cutin composition in antisense plants showed no alteration in these compounds. Analysis of the temporal and spatial activity of the *Arabidopsis* LTP promoter indicate that the protein may be involved in some aspect of phenylpropanoid metabolism. The antisense plants provide a system with which to test this and other hypotheses concerning the function of LTPs.

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CHAPTER 1

INTRODUCTION

Phospholipids are the major lipid component of most eukaryotic cell membranes. It has been estimated that eukaryotes may contain as many as 1000 chemically distinct phospholipids (Raetz, 1982) and the metabolic regulation and biological significance of the large number of lipid species is not well understood.. In eukaryotes, most enzymes responsible for lipid synthesis are on the cytoplasmic face of the endoplasmic reticulum (ER) (Alberts, *et al.*, 1989) and all plants also synthesize lipids in the chloroplast. Although lipid biosynthesis is restricted to a few organelles within a cell, lipids are found in all membrane systems of the cell, with different organelles containing specific and unique lipid compositions as well as an asymmetric distribution of lipid species across the bilayer.

The maintenance of a complex intracellular distribution of lipids must involve specific lipid transport mechanisms. However, the identification of such a transport mechanism in living cells has been difficult. There are three likely general mechanisms for lipid movement: (1) transport of molecules from one organelle to another by vesicle budding and fusion, (2) transport of molecules by lateral diffusion between organelles connected by membrane bridges, and (3) spontaneous or protein-mediated transport of lipid monomers through the cytosol. There has been evidence that vesicular transport is important in delivering phospholipids from their site of synthesis to the plasma membrane (Mills, *et al.*, 1984). However, this process has not been demonstrated to be important for delivering lipids to other membrane systems within a cell, so it is possible that other mechanisms are operational.

other mechanisms are operational.

Phospholipids rapidly migrate in the plane of the bilayer of biological membranes and in model membranes in the liquid crystalline state. By this process, phospholipids could move between organelles via transient or permanent interconnections between membranes. Associations between the ER and the chloroplasts have been observed in many plants including *Equisetum telmateia* (McLean, *et al.*, 1988), *Acer pseudoplantanus* and *Pinus pinea* (Wooding and Northcote, 1965), *Phaseolus vulgaris* (Whatley, *et al.*, 1991). Continuities have also been observed between the ER and other organelles, including the nucleus, the Golgi apparatus, the plasma membrane, and the outer mitochondrial membrane in the fern gametophyte of *Pteris vittata* L. (Crotty and Ledbetter, 1973).

Spontaneous transfer of native phospholipids does occur within a cell, but it occurs at rates too slow to account for the rate of membrane biogenesis within a cell (McLean and Phillips, 1982; Wirtz, 1982). Also spontaneous transfer is non-selective and would lead to a randomization of lipid components in the membranes. Protein mediated lipid transfer would require that the rate of membrane biogenesis within a cell is equal to the rate of diffusion of the lipid-protein complex through the cytosol. This, in fact, has been shown to be the case for the formation of certain organellar membranes. The specific and unique lipid composition of different cellular membranes could be maintained by this mechanism, as there could be a specificity as to which lipids particular proteins could bind and transport.

Protein mediated transport of phospholipids between membranes was first observed in 1968 by Wirtz and Zilversmit. The low level of exchange seen when rat liver microsomes and mitochondria were incubated together was significantly increased when a 105,000 g liver supernatant was added to the preparation. Subsequently, liver and beef heart preparations were shown to contain a factor which stimulated phospholipid exchange. This transport factor (LTP) was first partially purified from beef heart (Wirtz and Zilversmit, 1970). LTPs have since been purified from other mammalian tissues, yeast, bacteria, and plant tissue. Based on their ability to catalyze lipid transport *in vitro*, it has been suggested, despite the lack of experimental evidence, that LTPs carry out a similar role *in vivo*.

Mammalian tissue contains at least three different classes of LTPs. One class, purified initially from rat and bovine liver (Kamp, *et al.*, 1973; Lumb, et al., 1976), is highly specific for phosphatidylcholine (PC) and are called PC-transfer proteins (PC-TPs). The second class of proteins, the phosphatidylinositol (PI) transfer proteins (PI-TPs), isolated originally from bovine brain and heart (DiCorleto, *et al.*, 1979; Helmkamp, *et al.*, 1974), has a preference to bind PI, but can also transfer PC. An LTP purified from yeast (Szolderits, *et al.*, 1989) has a dual specificity closely resembling that of mammalian PI-TPs. However, the mammalian and yeast PI-TPs share no sequence similarity with respect to primary structure (Dickeson, *et al.*, 1989; Bankaitis, *et al.*, 1990). A phosphatidylserine (PS) transfer protein (PS-TP) has also been isolated from yeast (Paltauf and Daum, 1992). The

PS-TP can also transfer PE, cardiolipin, phosphatidic acid and ergosterol, but not PC or PI. No mammalian counterpart to the PS-TP has been identified. The third protein class, first purified from bovine and rat liver (Bloj and Zilversmit, 1977; Crain and Zilversmit, 1980), transfers a large variety of phospholipids, as well as cholesterol and neutral glycosphingolipids. These proteins are designated non-specific lipid transfer proteins (nsLTPs). The nsLTP from mammalian tissue has been shown to be identical to a protein which was previously designated sterol carrier protein 2 (Scallen, *et al.*, 1985a). This protein appears to function in several steps of cholesterol metabolism (Scallen, *et al.*, 1985b). A nsLTP has been purified from the bacteria, *Rhodopseudomonas sphaeroides* (Tai and Kaplan, 1985), from the filamentous fungus *Candida tropicalis* (Tan, *et al.*, 1990), and nsLTPs have been purified to homogeneity from several plant species.

Assay for lipid transfer activity

Lipid transfer activity is determined by measuring the stimulation of phospholipid transfer between natural membranes, such as microsomes, chloroplasts, or mitochondria, and artificially prepared, membranes, such as liposomes. One membrane, the donor membrane, is prepared to contain a radiolabeled lipid (such as [^{3}H]-phosphatidylcholine), and is then incubated with the non-labeled, acceptor membrane. After incubation of the membrane mixture with LTP, the donor and acceptor membranes are separated, and the appearance and increase of label in the acceptor membrane is analyzed. As it is rarely

possible to quantitatively separate donor and acceptor membranes, donor membranes are additionally labeled with a nontransferable tracer molecule (such as [^{14}C]-cholesteryl oleate). Such a tracer can monitor for cross contamination or incomplete recovery of membranes, and determination of the ^{3}H/^{14}C ratio in acceptor membranes then reflects the rate of lipid transfer.

Other, less common assays, involve the use of uni- and multilamellar vesicles which are separable by centrifugation, or the incubation of LTP with liposomes containing spin labeled lipids, whose movement can be followed by ESR spectroscopy (Nishida and Yamada, 1985).

Exchange versus net transport

The observation that phospholipid transport *in vitro* can be catalyzed by a protein raises the question of whether the movement takes place in both directions (exchange) or in one direction (net transport). There have been several reports in which an attempt has been made to elucidate the nature of this protein mediated transport, and the results have been somewhat disparate. In the presence of bovine PC-TP, net transfer of PC was observed to acceptor vesicles prepared from PE and phosphatidate (Wirtz, *et al*, 1980). However, the concentration of LTP used in this experiment was much higher than physiological levels and when lower concentrations were used, only PC exchange was observed (Helmkamp, 1980). Crain and Zilversmit (1980) demonstrated that a bovine liver nsltp catalyzed net transfer of PC and PI from PC/PI multilamellar vesicles to intact

or delipidated human high density lipoproteins. Under identical conditions, however, it was observed that a bovine PC-TP and a bovine heart PI/PC-TP catalyzed exchange of PC and PI.

Purification of non-specific lipid transfer proteins from plants

It was shown by Kader (1975) that soluble extracts from potato tubers contained a protein component which enhanced the movement of lipids between microsomal fractions and mitochondria isolated from cauliflower and potato tuber. Since that time, LTPs have been purified to homogeneity from maize seedlings (Douady, *et al*, 1982), spinach leaves (Kader, *et al.*, 1984), and castor bean seedlings (Takishima, *et al.*, 1986; Watanbe, *et al*, 1986), and are quite well characterized at the biochemical level. A barley protein, first classified as a probable amylase/proteinase inhibitor (Mundy and Rogers, 1986), was subsequently identified as an LTP (Bernhard and Somerville, 1989; Breu, et al, 1989).

Biochemical properties

Specificity of plant lipid transfer proteins As previously suggested, all LTPs which have been isolated and extensively characterized from plants are non-specific; i.e., they transfer a large variety of phospholipids in *vitro* assays (Table 1-1). The maize and barley LTPs have been shown to transfer PC, PI, and phosphatidylethanolamine (PE) (Douday, *et al.*, 1985; Kader, 1985), and the

BIOCHEMICAL PROPERTIES OF PLANT NON-SPECIFIC LIPID TRANSFER PROTEINS

Source	Molecular Mass (kD)	Isoelectric Point	Substrate Specificity	Reference
Barley	10	-	PC,PI,PE	Kader, 1985
Castor[1]	9.3	10.5	PC,PI,PE, PG,MGDG	Tsuboi, *et al.*, 1991
Maize	9.1	8.8	PC,PI,PE	Douady, *et al.*, 1985
Spinach	9.0	9.0	PC,PI,PE,PG, MGDG,fatty acids	Kader, *et al.*, 1984; Tchang, *et al.*, 1988
Tomato	8.85	9.0	-	Torres-Schuman, *et al.*, 1992
Wheat	9.0	-	-	Diercyk,*et al.*, 1992

Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; MGDG, monogalactosyldiacylglycerol

[1]The isoforms of castor have similar biochemical properties.

proteins isolated from spinach and castor have been shown to transfer PC, PI, PE, phoshphatidylglycerol (PG), and monogalactosyldiacylglycerol (MGDG) (Kader, *et al.*, 1984; Tchang, *et al.*, 1988; Tsuboi, *et al.*, 1991). Four isoforms of LTP, A, B, C, and D have been isolated from castor bean seedlings, and the substrate specificities are similar among isoforms (Tsuboi, *et al.*, 1991).

Besides transferring lipid molecules, it has been demonstrated that lipid transfer proteins can bind fatty acids. Rickers, *et al.* (1984) isolated a fatty acid binding protein from onion leaves and it was subsequently shown that this protein had the ability to transfer PC in an *in vitro* assay (Arondel, *et al.*, 1989). It was later demonstrated that the spinach LTP has the ability to bind long chain fatty acids (oleic and linoleic) and oleoyl-CoA (Rickers, *et al.*, 1985). The castor bean isoform nsltp-A also has a binding capacity for oleic acid and oleoyl CoA (Yamada, *et al.*, 1990)

Molecular mass The apparent molecular mass of plant lipid transfer proteins, as determined by SDS-polyacrylamide gel electrophoresis and gel filtration is approximately 9 kDa (Table 1-1). These values correspond well with the molecular masses determined from protein sequences (Bouillon, *et al.*, 1987) and are close to those established for nsltps from animal sources, which range from 11.2 to 14.5 kDa (Helmkamp, 1990). A dimeric form of maize LTP (M, 18,000 ± 1000) has been observed when electrophoresis is carried out under non-reducing conditions (Douady, *et al.*, 1985). Specific LTPs from mammalian and yeast tissue

are larger, ranging from 25-35 Kda (Helmkamp, 1990).

Isoelectric point Determination of Pi by chromatofocusing or isoelectric focusing shows that plant nsLTPs are basic (Table 1-1). Maize, spinach, and castor proteins have pIs of 8.8, 9.0, and 10.5, respectively. A 10:3 proportion of basic residues versus acidic residues gives a tomato LTP a calculated isoelectric point value of 8.85 (Torres-Schumann, *et al.*, 1992). Acidic proteins (pI of about 4.5) have also been detected in spinach leaf and castor bean seedlings (Tanaka and Yamada, 1979), but these proteins have not been well characterized.

Stability Plant nsLTPs are very stable proteins. The castor protein isolated by Watanbe and Yamada (1986) retained transfer activity after storage for one month at 4°C, or 30 minutes at 90°C. Similarly, the maize nsLTP is stable for one month at 4°C and it retains 50% of its transfer activity after 30 minutes at 95°C (Arondel and Kader, 1990).

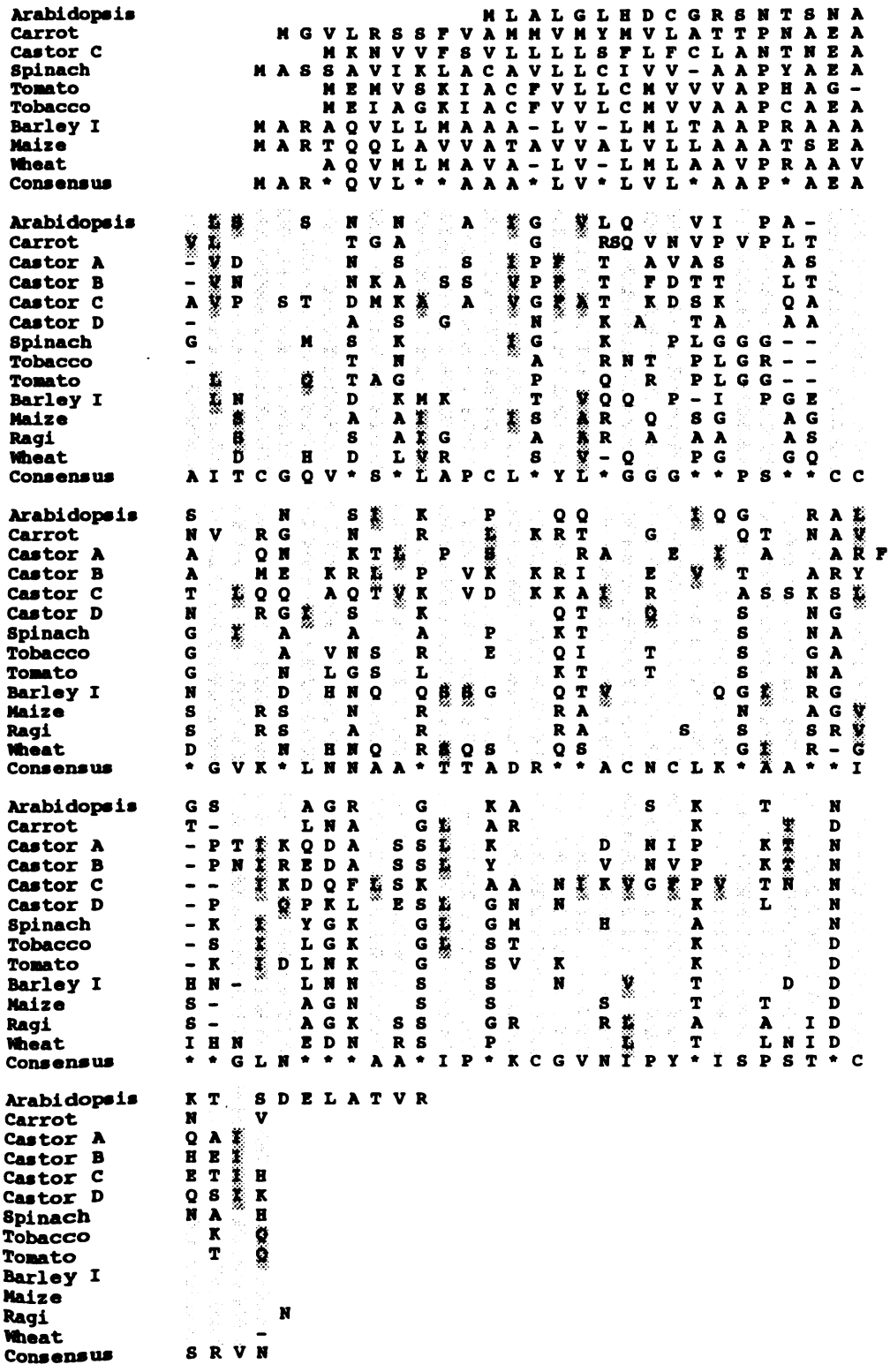
Abundance Although specific LTPs from mammalian sources are of relatively low abundance (0.05% - 0.25%) (Helmkamp, 1990), plant nsLTPs have been reported to be highly abundant. Using immunological techniques, Grosbois *et al.* (1987) determined that maize LTP constituted 4.1% of the total soluble protein in crude extracts of maize seedlings and it has been reported that LTP accounts for 10% of the total soluble protein at certain stages of maize seedling

development (Grosbois, *et al.*, 1989). The *Arabidopsis* LTP, however, is not so abundant as it constitutes no greater than 0.1% of the total soluble protein in crude leaf extracts (Thoma, S., unpublished data).

Protein structure

Amino acid sequence for the nsLTPs from maize seedlings (Dubois, *et al.*, 1987; Tchang, *et al.*, 1988), spinach leaves (Bouillon, *et al.*, 1987; Bernhard, *et al.*, 1991), castor bean seedlings (Takishima, *et al.*, 1986), barley caryopses (Svensson, *et al.*, 1986), sunflower (Arondel, *et al.*, 1990), carrot (Sterk, *et al.*, 1991) and wheat (Dieryck, *et al.*, 1992) have been determined by Edman degradation of the purified protein or by analysis of the cDNA corresponding to the protein. These plant LTPs, while lacking any obvious sequence homology to LTPs from yeast, bacteria, or mammalian cells, share a high amino acid similarity among themselves (Fig. 1-1). This is especially noticeable in the central portion of the protein, where hydropathy profiles indicate the presence of a deep hydrophilic region surrounded by two hydrophobic regions (Arondel and Kader, 1990; Yamada, 1992). It has been hypothesized that the hydrophobic regions are involved in binding to the acyl chains of a lipid molecule, and the hydrophilic region may associate with the polar region of the lipid molecule (Takishima, *et al.*, 1988). These proteins also exhibit a hydrophobic N-terminal end and it has been suggested that this region of the protein may be important for lipid-membrane interactions (Arondel and Kader, 1990). Secondary structure analysis of a barley

Figure 1-1. Comparison of the amino acid sequences of plant non-specific lipid transfer proteins. Amino acids which are identical to the consensus sequence are indicated by a shaded gray area (▒). Conserved substitutions are represented by the single letter amino acid designation which is highlighted with gray.


Arabidopsis
Carrot
Castor C
Spinach
Tomato
Tobacco
Barley I
Maize
Wheat
Consensus
Arabidopsis
Carrot
Castor A
Castor B
Castor C
Castor D
Spinach
Tobacco
Tomato
Barley I
Maize
Ragi
Wheat
Consensus
Arabidopsis
Carrot
Castor A
Castor B
Castor C
Castor D
Spinach
Tobacco
Tomato
Barley I
Maize
Ragi
Wheat
Consensus
Arabidopsis
Carrot
Castor A
Castor B
Castor C
Castor D
Spinach
Tobacco
Tomato
Barley I
Maize
Ragi
Wheat
Consensus
Arabidopsis
Carrot
Castor A
Castor B
Castor C
Castor D
Spinach
Tobacco
Tomato
Barley I
Maize
Ragi
Wheat
Consensus

LTP (Madrid and von Wettstein, 1991), indicates that the protein has three amphiphilic regions which could form a pocket for the binding of phosphoglycerides, and it is proposed that the hydrophilic surface of the protein allows for the solubility of the lipid-protein complex in the cytosol. All plant nsLTPs characterized to date contain 8 cysteine residues, and the positions of these residues are completely conserved. It has been demonstrated that the cysteine residues in the castor bean protein form four disulphide bridges (Takishima, *et al.*, 1988).

Occurrence of isoforms

There have been reports of multiple isoforms of LTPs in a variety of plant species. Four isoforms of LTP have been isolated from castor bean seedlings, and they have been shown to be differentially expressed in a tissue specific manner (Tsuboi, *et al.*, 1991). Two peaks of activity observed during purification of maize LTP suggest the presence of at least two isoforms in this plant (Arondel, *et al.*, 1990). This result was confirmed by Southern analysis of maize genomic DNA which indicates the presence of several maize LTP genes (Tchang, *et al.*, 1989). Purification of wheat LTP led to the isolation of three proteins which had PC transfer activity *in vitro*, two with a molecular mass of 9 kD and the other 7 kD (Dieryck, *et al.*, 1992). The presence of isoforms in spinach tissue was suggested by the observation that the protein separated into two fractions upon purification (Kader, *et al.*, 1984). However, high stringency Southern analysis indicates the

presence of only one gene (Bernhard, *et al.*, 1991; chapter 2 of this dissertation) suggesting that the isoforms are not closely related at the amino acid level. Southern analysis of tomato DNA under high stringency conditions was consistent with the presence of only one gene, but low stringency hybridization indicates the presence of several LTP-like genes (Torres-Schumann, *et al.*, 1992). Southern analysis shows the presence of a single gene in the carrot genome (Sterk, *et al.*, 1991). Two different LTPs have been found in tobacco. The first was isolated as an anther specific protein is found to be expressed predominantly in tapetal cells (Koltunow, *et al.*, 1990) and the other is expressed in all aerial tissues of the plant, but was not expressed in tapetal cells (Fleming, *et al.*, 1992). Barley also encodes at least two LTPs. The first was originally identified as an amylase/proteinase inhibitor based on sequence homology (Mundy and Rogers, 1986) and the other has more recently been identified (R. Kalla, personal communication).

Localization of plant nsLTPs

Because of their proposed function of intracellular lipid transfer, it has long been assumed that LTPs were located in the cytosol (Arondel and Kader, 1990; Helmkamp, 1990). However, recent evidence indicates that nsLTPs are not located in the cytoplasm. It has been reported that the maize LTP has an amino terminal extension and is synthesized on membrane-bound polysomes (Tchang, *et al.*, 1988; Vergnolle, *et al.*, 1988) and that spinach and barley LTPs contain signal peptides and are cotranslationally inserted into microsomal membranes

(Bernhard, *et al.*, 1991; chapter 2 of this dissertation; Madrid, 1991). Since these proteins lack the endoplasmic retention signal, KDEL (Munro and Pelham, 1987), they would be expected to be secreted or targeted to a specific organelle. In support of these expectations, Mundy and Rogers (1986) reported that the barley protein which was subsequently identified as an LTP (Breu, *et al.*, 1989) was found in aleurone cell culture medium and Sterk, *et al.* (1991) demonstrated that a carrot LTP was secreted by embryogenic cell cultures. Using immunogold labeling and light microscopy, Sossountzov, *et al.* (1991), have shown that a maize LTP is localized to the periphery of the epidermal cells in maize coleoptile and suggest that the labeling is associated with the plasma membrane. It has more recently been demonstrated (Thoma, *et al.*, 1993; chapter 3 of this dissertation), using immunocytochemical labeling at the ultrastructural level, that an *Arabidopsis* LTP is localized to the cell wall, and that it is mainly seen in epidermal cell walls.

Scope of dissertation

Although LTPs have a demonstrated ability to catalyze lipid transfer between membranes *in vitro*, it has been difficult to assign an *in vivo* function to these proteins. A yeast PI-TP is the only LTP to which the *in vitro* activity correlates to a function *in vivo* (Bankaitis, *et al.*, 1990). It appears that this protein is involved in elevating PI/PC ratios of yeast Golgi membranes by an unknown mechanism (Cleves, *et al.*, 1991). The nsLTPs from plants, however, lack sequence homology to the yeast PI-TP and are likely to have a different biological role.

The original objective of the research described in this dissertation was to examine the possible role of nsLTPs in membrane biogenesis. As a first step, a cDNA corresponding to a spinach nsLTP was cloned. Analysis of this clone revealed the presence of a signal peptide, and was subsequently shown to be cotranslationally inserted into microsomal membranes (Chapter 2). These observations raised questions about the localization of LTP and thus, about the role of LTP within the cell. To address these questions, antibodies raised against a protein A:*Arabidopsis* LTP fusion protein were used to localize the protein by immunological techniques at the ultrastructural level (Chapter 3) and transgenic plants containing an *Arabidopsis* LTP promoter - ß-glucuronidase fusion were analyzed (Chapter 4). Finally, in an attempt to determine the role of this protein within a plant, *Arabidopsis* plants containing a very reduced level of LTP due to the presence of the *Arabidopsis* LTP cDNA in reverse orientation behind the Cauliflower Mosaic Virus (CaMV) 35S promoter were produced and analyzed (Chapter 5). A summary of the conclusions of this work and suggestions for future research are discussed in chapter 6.

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CHAPTER 2

ISOLATION OF A CDNA CLONE FOR A SPINACH LIPID TRANSFER PROTEIN AND EVIDENCE THAT THE PROTEIN IS SYNTHESIZED BY THE SECRETORY PATHWAY

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ABSTRACT

A cDNA clone encoding a non-specific lipid transfer protein from spinach was isolated by probing a library with synthetic oligonucleotides based on the amino acid sequence of the protein. Determination of the DNA sequence indicated a 354-nucleotide open reading frame which encodes a 118-amino acid residue polypeptide. The first 26 amino acids of the open reading frame, which are not present in the mature protein, have all the characteristics of a signal sequence which is normally associated with the synthesis of membrane proteins or secreted proteins. *In vitro* transcription of the cDNA and translation in the presence of canine pancreatic microsomes or microsomes from cultured maize endosperm cells indicated that proteolytic processing of the preprotein to the mature form was associated with cotranslational insertion into the microsomal membranes. Since there is no known mechanism by which the polypeptide could be transferred from the microsomal membranes to the cytoplasm, the proposed role of this protein in catalyzing lipid transfer between intracellular membranes is in doubt.

Although the lipid transfer protein is one of the most abundant proteins in leaf cells, the results of genomic Southern analysis were consistent with the presence of only one gene. Analysis of the level of mRNA by Northern blotting indicated that the transcript was several-fold more abundant than an actin transcript in leaf and petiole tissue, but was present in roots at less than 1% of the level in petioles.

INTRODUCTION

Lipid transfer proteins, which have been isolated from animals, yeast, plants and bacteria, are characterized by their ability to catalyze exchange of lipids between natural or artificial membranes *in vitro* (Kader, 1985; Helmkamp, 1986; Wirtz and Gadella, 1990). Under conditions in which the donor membrane is actively engaged in lipid synthesis, plant lipid transfer proteins have also been shown to catalyze net transfer from the donor to acceptor membrane (Miguel, *et al.*, 1987). On this basis, it has been proposed that they participate in the transfer of lipids between membranes in intact cells (Miguel, *et al.*, 1987). However, this family of proteins is relatively poorly characterized in several respects, and a role in intracellular lipid transport is not established (Bishop and Bell, 1988).

In higher plants, both specific and non-specific lipid transfer proteins have been characterized (Kader, 1985; Watanabe and Yamada, 1986). The basic lipid transfer proteins from maize seedlings (Douady, *et al.*, 1982), spinach leaves (Kader, *et al.*, 1984) and castor bean seedlings (Watanabe and Yamada, 1986) are non-specific. All non-specific plant lipid transfer proteins are soluble proteins which may account for as much as 4% of the total soluble protein (Kader, 1985). The intracellular location of the lipid transfer proteins has not been established but they are located outside the chloroplast (Schwitzguebel and Siegenthaler, 1985) and have been thought to be cytosolic. The most thoroughly characterized proteins have a pI of about 9 and a molecular mass of about 9000 Da (Kader, *et al.*, 1984; Watanabe and Yamada, 1986). The amino acid sequences have been

determined for the spinach leaf (Bouillon, *et al.*, 1987) and castor seedling proteins (Takishima, *et al.*, 1986). In addition, on the basis of amino acid sequence identity to the known lipid transfer proteins, several polypeptides from barley and finger millet, which were originally described as probable amylase inhibitors (Mundy and Rogers, 1986), were identified as lipid transfer proteins (Bernhard and Somerville, 1989; Breu, *et al.*, 1989). Recently, a cDNA of the maize lipid transfer protein has been characterized (Tchang, *et al.*, 1986). Comparison of the deduced amino acid sequences of cDNA clones encoding the maize and barley lipid transfer proteins with the directly determined amino terminal sequences of the mature proteins indicated that these proteins are synthesized as precursors containing 27 or 25 additional N-terminal amino acids, respectively.

In order to establish conditions for the analysis of the role of lipid transfer proteins by genetic methods, a full length cDNA clone for the non-specific lipid transfer protein from spinach leaves has been isolated and characterized. I have characterized the level of mRNA abundance in various tissues and the number of closely related genes in the spinach genome was examined. In addition, I describe here the results of experiments indicating that synthesis of the mature protein involves processing of a pre-protein by the secretory pathway.

EXPERIMENTAL PROCEDURES

Materials - A λgt11 cDNA library derived from mRNA of spinach leaves (*Spinacia oleracea* L. American Hybrid 424), was obtained from W. L. Ogren

(United States Department of Agriculture, Urbana, IL). Plasmid pSP65AB30, which carries a cDNA encoding the light harvesting chlorophyll a/b binding protein from *Lemna gibba* under transcriptional control of the SP6 promoter (Kohorn, *et al.*, 1986) was obtained from E. Tobin (UCLA, Los Angeles, CA). Plasmid pSPBP1 encoding a cDNA for bovine prolactin under transcriptional control of the SP6 promoter was obtained from V.R. Lingappa (UCSF, San Francisco, CA). Plasmid pSAc3 carrying an actin gene from soybean (Shah, *et al.*, 1982) was obtained from R. Meagher (University of Georgia, Athens, GA). The plasmid pBluescript (BS^+) was purchased from Stratagene (San Diego, CA). Oligonucleotides were synthesized by the phosphoramidite method on an Applied Biosystems 380A instrument.

A preparation of microsomal membranes suitable for *in vitro* translocation and processing of precursor proteins was generously provided by J. Miernyk (US Department of Agriculture, Peoria, IL). The microsomes were prepared from maize endosperm cultures (Miernyk, 1987) by a modification (R.G. Shatters Jr. and J.A. Miernyk, manuscript in preparation) of the method of Burr and Burr (1981).

Plaque Screening - The cDNA library was plated on *Escherichia coli* Y1090 and nitrocellulose plaques lifts were screened with the oligonucleotide mixtures SLTP-1 (ATGTG(C/T)GGNGTNCA(C/T)AT) and SLTP-2 (AA(A/G)GGNAT(T/C)AA(T/C)TA-(T/C)GG) which were labeled to an average specific activity of 10^9 cpm μg^{-1} with [γ-^{32}P]ATP (3000 Ci $mmol^{-1}$) and T4

polynucleotide kinase (Maniatis, *et al.*, 1982). The filters were prehybridized 3 to 5 h at 42°C for SLTP-1 and at 39°C for SLTP-2, in 6 x SSC[2], 50 mM $NaPO_4$ (pH 6.8), 5 x Denhardt's solution and 0.5 mg ml^{-1} sonicated herring DNA (Sigma). The hybridization was carried out at the same temperatures for 36 h with the addition of 2 pmol of the appropriate oligonucleotide mixture. The filters were washed in 4 x SSC at the same temperature as the hybridization, with 4 changes of the washing buffer at 30 min intervals.

Nucleic Acid Manipulations - RNA and DNA was extracted from 6 week old spinach by grinding 5 g of tissue in liquid N_2, then stirring for 15 min at 60°C in a mixture of 50 ml of 100 mM Tris-HCl pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5% (w/v) SDS, 2% (v/v) 2-mercaptoethanol and 50 ml of phenol. The mixture was then extracted once with 100 ml of chloroform/isoamyl alcohol (24:1), once with phenol/chloroform/isoamyl alcohol (24:24:1) and the nucleic acids recovered by ethanol precipitation. The pellet was resuspended in 20 ml water, 1 volume of 4 M LiCl was added and after incubation for 16 h at -20°C the RNA was pelleted by centrifugation at 12,000 x g for 60 min. The RNA pellet was washed with 70% ethanol. DNA was isolated from the supernatant of the LiCl precipitation by adding 2 volumes of ethanol and centrifuging at 15,000 x g for 60 min, then purified by CsCl centrifugation.

Fragments to be sequenced were subcloned into the appropriate restriction site of pBluescript and sequenced by the chain termination method using

Sequenase (US Biochemicals).

For Southern analysis DNA was restricted and resolved by electrophoresis on a 0.8 % agarose gel (10 μg per lane). Following depurination in 0.25 M HCl and denaturation in 0.5 M NaOH, 1.5 M NaCl the DNA was transferred in 0.25 M NaOH, 1.5 M NaCl to nylon membrane (Hybond N, Amersham). The membrane was prehybridized 3 to 5 h at 50°C in 6 x SSC, 5 x Denhardt's solution and 1 mg ml^{-1} sonicated herring DNA. The hybridization was carried out at 50°C for 10 h with the addition of 0.2 μg of the purified insert from the plasmid pWB2 labelled with [α-^{32}P]dCTP by random priming (Feinberg and Vogelstein, 1983) to an average specific activity of 5 x 10^8 cpm μg^{-1}. The membranes were washed once with 1 x SSC, 0.2% SDS at 50°C and 3 times with 0.1 x SSC, 0.2% SDS at the same temperature.

For Northern analysis 20 μg RNA was resolved by electrophoresis in an agarose gel containing formaldehyde (Maniatis, *et al.*, 1982). After the transfer (in 10 x SSC) to nitrocellulose, the blot was prehybridized at 42°C in 5 x SSC, 5 x Denhardt's solution, 50 mM $NaHPO_4$ (pH 7.0), 0.01% SDS, 50% deionized formamide and 1 mg ml^{-1} sonicated herring DNA. The hybridization was carried out at the same temperatures for 10 h with the addition of 0.4 μg of the purified insert of the plasmid pWB2 labelled with [α-^{32}P]dCTP by random priming. The membranes were washed twice with 2 x SSC, 0.1% SDS at 23°C, twice with 2 x SSC, 0.1% SDS at 60°C and once with 0.2 x SSC, 0.1% SDS at 60°C.

In vitro Transcription and Translation - Transcripts were prepared from linearized plasmids with T3 RNA polymerase in the presence of 500 μM $m^7G(5')ppp(5')G$ (Pharmacia) and 2.5 mM of each ribonucleotide triphosphate at 38°C for 1 h. The reaction was terminated by addition of 1 U of DNAse per μg of template DNA. After 10 min the reaction was extracted with chloroform/phenol (1:1), then with chloroform, adjusted to 0.3 M sodium acetate and RNA recovered by ethanol precipitation.

Translation assays containing 0.5 μg RNA, 15 μl of wheat germ extract (Promega), 15 μCi ^{35}S-methionine (1149 Ci/mmol; Amersham) in a final volume of 30 μl were incubated 1 h at 30°C. No additional KOAc or MgOAc was required for maximal rates of translation of transcripts from pWB2 or pSP65AB30. Maximal rates of translation of transcripts from pSPBP1 were obtained following addition of 33 mM KOAc. For some experiments 3 μl of canine pancreatic microsomes (Promega), or 8 μl (21.4 μg of protein) of microsomes from cultured cells of maize endosperm tissue, were added to the translation reactions. The translation products were resolved electrophoretically by loading equal amounts of incorporated radioactivity on 10 to 20% gradient SDS polyacrylamide gels. The resolved proteins were electrophoretically transferred to nitrocellulose then the filters were autoradiographed.

Protease Protection - Translation reactions were terminated by addition of 1 μl of a solution containing 3 mM cycloheximide and 0.12 M methionine, and

divided into three identical samples. One sample was left untreated as a control. $CaCl_2$ (10 mM) and proteinase K (1 mg ml^{-1}) were added the second sample. The third aliquot was adjusted to contain 1% Triton X-100 before addition of the protease. The reactions were incubated at 24°C for 1 h and the treatments were terminated by addition of 0.1 volume of 100 mM phenethylmethyl sulfonyl fluoride in isopropanol.

RESULTS

Isolation and Sequence Analysis of a cDNA Clone - On the basis of the amino acid sequence of the lipid transfer protein of spinach (Bouillon, *et al.*, 1987) two oligonucleotide mixtures composed of 64 (SLTP-1) and 96 (SLTP-2) different 17-mers were prepared, which contained all possible DNA sequences which could encode the corresponding regions of the protein sequence. Screening of a λgt11 cDNA library derived from spinach leaf poly(A^+) RNA resulted in the recovery of two positive plaques from among approximately 75,000 screened. Both were identical and contained two EcoRI fragments of 580 and 730 bp, respectively. These inserts were subcloned into the EcoRI site of pBluescript to produce the plasmids pWB2 and pWB3 and the DNA sequence of the inserts in both plasmids was determined. The insert in plasmid pWB2 was found to encode the spinach lipid transfer protein. The insert in pWB3, which also had a poly-(A^+) sequence at one end, appeared to be a fragment of an unrelated cDNA which could not be

identified by sequence homology to any previously determined DNA or protein sequence and was not characterized further.

The composite nucleotide and deduced amino acid sequences of pWB2 are shown in Fig 2-1. The entire cDNA was 564 nucleotides long and contained one open reading frame of 354 bp which began 13 bp from the 5' end and terminated 197 bp from the 3' poly(A) tail. The open reading frame encoded a polypeptide of 118 amino acids. The carboxy terminal 92 residues of the open reading frame were almost identical to the directly determined amino acid sequence of the mature protein (Bouillon, *et al.*, 1987). The only differences were that the deduced amino acid sequence had cysteines rather than serines at residues 27 and 28. Cysteines are also found at these positions in all other homologous proteins (Bernhard and Somerville, 1989). The deduced amino acid sequence also contained 26 amino terminal residues which were not found on the mature polypeptide. This observation is consistent with evidence from the analysis of the maize (Tchang, *et al.*, 1988) and barley (Mundy and Rogers, 1986; Bernhard and Somerville, 1989) genes indicating that these polypeptides are produced by cleavage of a preprotein.

RNA Analysis - In order to assess the size of the transcript encoding the spinach lipid transfer protein, and to evaluate the tissue specificity of gene expression, the cDNA clone was used to probe a Northern blot containing spinach RNA from various tissues (Fig. 2-2A). The results of this experiment indicated that

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TTGCATTATA TTT  ATG GCT AGC TCC GCT GTT ATC AAG TTA GCT TGT   46
                 M   A   S   S   A   V   I   K   L   A   C  -16

GCA GTC CTG TTG TGC ATC GTG GTC GCT GCA CCA TAC GCT GAA GCA   91
 A   V   L   L   C   I   V   V   A   A   P   Y   A   E   A   -1

GGT ATA ACT TGT GGG ATG GTT TCA AGC AAA CTT GCT CCT TGC ATT  136
 G   I   T   C   G   M   V   S   S   K   L   A   P   C   I   15

GGG TAC CTT AAA GGA GGC CCC TTG GGC GGT GGT TGC TGT GGT GGA  181
 G   Y   L   K   G   G   P   L   G   G   G   C   C   G   G   30

ATT AAG GCC CTG AAC GCG GCA GCT GCC ACC ACT CCT GAC AGG AAA  226
 I   K   A   L   N   A   A   A   A   T   T   P   D   R   K   45

ACT GCA TGC AAT TGC CTC AAA AGT GCT GCT AAT GCC ATT AAG GGA  271
 T   A   C   N   C   L   K   S   A   A   N   A   I   K   G   60

ATC AAC TAC GGA AAG GCT GCT GGT CTC CCT GGT ATG TGT GGC GTC  316
 I   N   Y   G   K   A   A   G   L   P   G   M   C   G   V   75

CAT ATT CCC TAC GCC ATT AGC CCC AGC ACC AAC TGC AAC GCC GTC  361
 H   I   P   Y   A   I   S   P   S   T   N   C   N   A   V   90

CAC TAA  ACCGCAAATGTTATAACAAAAATGGAAGATGGAGCTACATAGGAGTGGCC  417
 H   *

CAGTTACTAAGCTCGTAGAGTGTATGATAATAAAAGAAGAGAGATCATCTTTGCCAAGT  476

CGCTAGCTTGTATTTCTTGTTTCATGTATTATTGCAACTTTTCTATTACTTTTCGGGTT  535

ACAAATATCCTAATATTACCAAAAAAAAA                                564
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Figure 2-1. Composite nucleotide sequence of the cDNA for spinach lipid transfer protein and the deduced amino acid sequence. The amino acids are numbered from the glycine residue found at the amino terminus of the mature protein (Bouillon, *et al.*, 1987). The regions of sequence homologous to the oligonucleotide probes used to isolate the gene are underlined. The cDNA was isolated by Werner Bernhard. Sequencing was carried out by Werner Berhard and Jose Botella.

the probe hybridized to an mRNA of approximately 700 bp in RNA preparations from leaves and petioles. Since this is similar to the size of the cDNA clone in pWB2, it appears that the insert in pWB2 represents a nearly full-length cDNA. There was a striking difference in expression of the mRNA in green tissues versus root tissue, where accumulation of the lipid transfer protein mRNA was not readily apparent. On prolonged exposure of the filter a faint signal could be observed in the lane of root RNA (results not presented). This signal was estimated to be approximately 100-fold less abundant than the leaf signal. In order to verify that the preparation of RNA from the various tissues were of comparable quality, the filter was stripped and reprobed with an actin gene which was previously shown to hybridize to a similar extent to RNA from roots, shoots and hypocotyls of soybean (Hightower and Meagher, 1985). The presence of hybridization signals of similar intensity in the root and petiole lanes when probed with the actin gene (Fig 2-2B) verified that the two preparations of RNA were of similar quality and quantity.

Southern Analysis - Two protein fractions with lipid transfer activity have been reported for spinach (Kader, *et al.*, 1984), suggesting the presence of isoforms of lipid transfer protein. To investigate the number of homologous genes present in the spinach genome, Southern analysis was performed with genomic DNA cut with several restriction enzymes which do not have recognition sites in the cDNA sequence. For 5 of the 6 restriction enzymes used, the cDNA probe hybridized to only one fragment, and in 4 of these cases the fragment was less

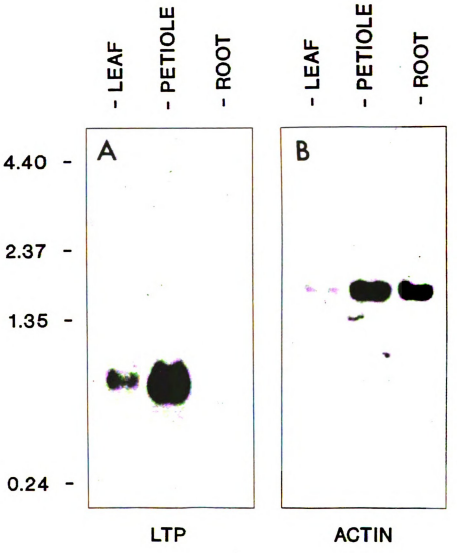

Figure 2-2. Northern blot of total RNA from spinach probed with the cDNA of spinach lipid transfer protein labelled to a specific activity of 2 x 10^9 dpm/μg DNA (A) then stripped and reprobed with an actin cDNA labeled to a specific activity of 3 x 10^9 dpm/μg DNA (B). Twenty μg of RNA was loaded in each lane. The filter in A was exposed 2 days, the filter in B was exposed 5 days. The size (in kb) and location of molecular weight markers is indicated at the left of the figure.

than 4 kb in size (Fig. 2-3). Thus, it appears likely that only one gene encoding the lipid transfer protein is present in the spinach genome. The presence of two bands in the lane containing DNA restricted with DraI suggests the presence of at least one intron in the genomic sequence. In view of the very high abundance of the protein in leaf tissues, the mechanism by which a single gene appears to regulate accumulation of the protein may merit further investigation with respect to protein and mRNA turnover and rate of transcription.

In Vitro Translation and Processing - The sequence of the 26 amino-terminal residues of the deduced amino acid sequence of the spinach lipid transfer protein contained many of the features associated with signal sequences (Watson, 1984; von Heijne, 1985). The general characteristics of signal peptides are: (1) they are 20 to 40 amino acids long, (2) there is a charged residue within the first 5 amino acids in the amino-terminal direction from the cleavage site and this is followed by a core of at least nine hydrophobic residues, (3) A helix-breaking residue (glycine or proline) frequently occurs 4 to 8 residues before the cleavage site. The most stringent requirement is that an alanine residue occurs at the minus-1 and minus-3 positions. The prepeptide of the spinach lipid transfer protein satisfies all of these criteria.

The role of the amino-terminal sequence was tested by transcribing and translating the cDNA *in vitro* under conditions which lead to processing of signal peptides by the secretory pathway (Walter and Lingappa, 1986). When transcripts

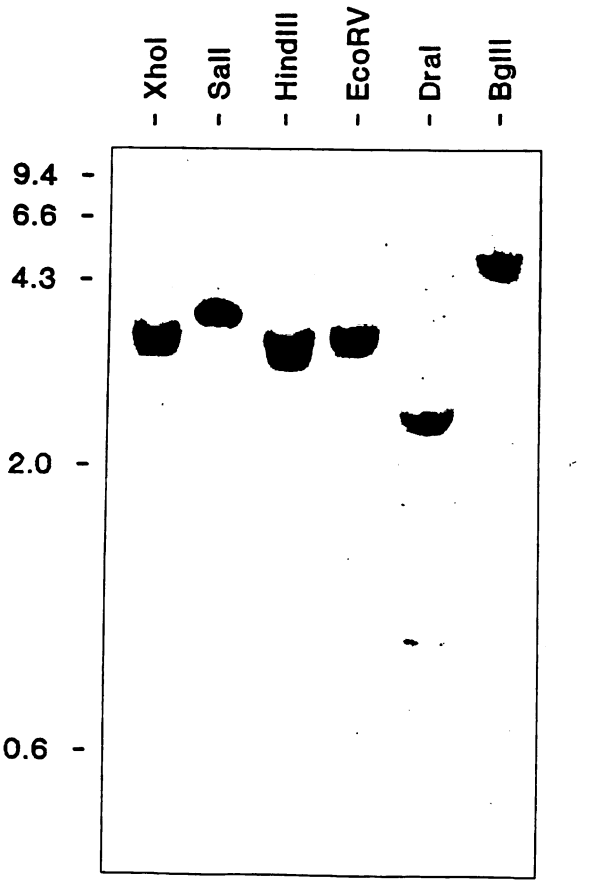

Figure 2-3. Southern blot of total spinach DNA probed with the cDNA of spinach lipid transfer protein. The size (in kb) and location of molecular weight markers is indicated at the right of the figure. Southern analysis was carried out by Werner Bernhard.

from the cDNA were translated without addition of microsomal membranes (Fig. 2-4D), or when the dog microsomal membranes were added after translation was complete (Fig. 2-4F), the translation product was of the size expected from the open reading frame. By contrast, when dog microsomal membranes were present during translation, a translation product of the size expected for the mature polypeptide accumulated (Fig. 2-4E). Identical results were obtained with the positive control, prolactin (Fig. 2-4A-C), whereas the presence of dog microsomes had no effect on the size of the chlorophyll a/b binding protein (Fig. 2-4G,H) which is inserted into chloroplast membranes by a different mechanism. Replacement of dog microsomes with maize microsomal membranes resulted in processing of the primary translation product to a processed form of the same size as that obtained with the dog microsomes (Fig. 2-5). Thus, the amino termius of the preprotein functions as a signal peptide in both plants and animals.

In order to determine if the lipid transfer protein was cotranslationally inserted into the microsomal membranes, the protease sensitivity of the translation products was assessed by treating the translation reactions with proteinase K, then inactivating the proteinase with phenethylmethylsulfonyl fluoride prior to SDS-PAGE. When translated in the absence of microsomes, all of the translation products from the lipid transfer gene and the control gene (prolactin) were protease sensitive (Fig. 2-6B,G). By contrast, when transcripts from the lipid transfer protein and prolactin genes were translated in the presence of dog microsomes, the low molecular weight product was protected from proteolysis but

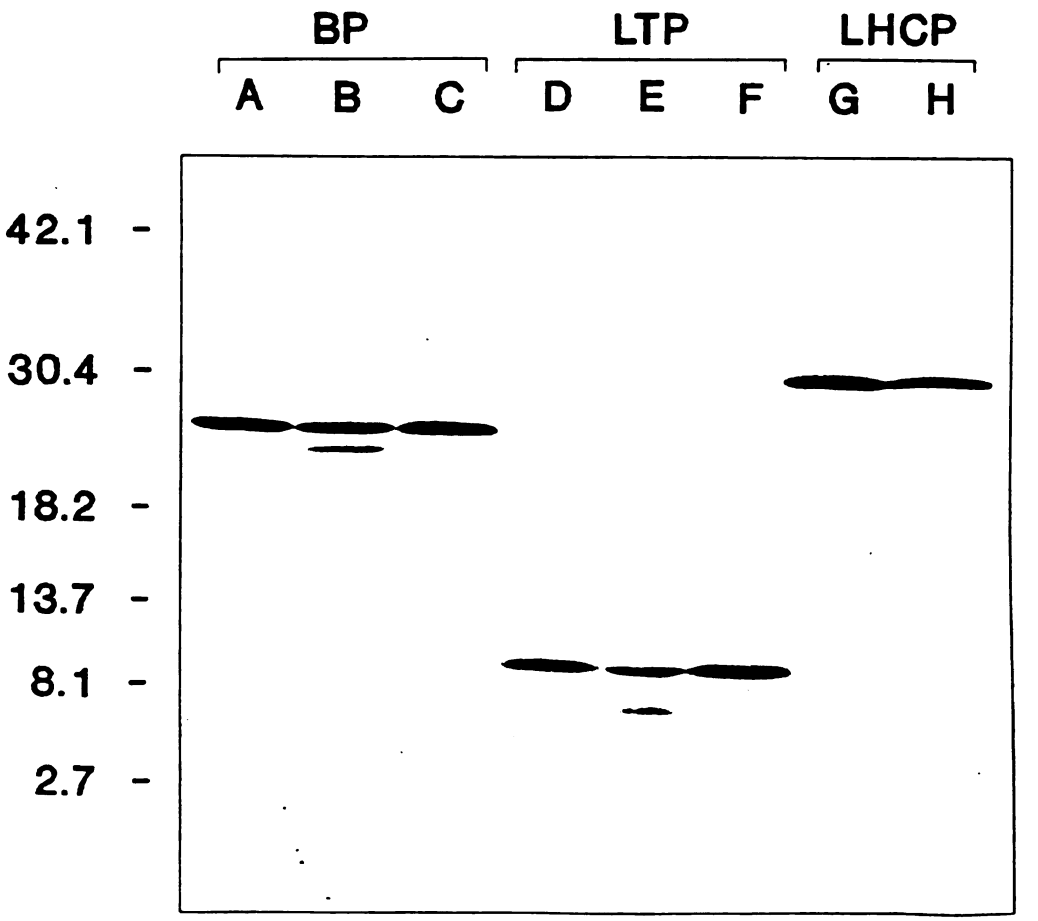

Figure 2-4. Processing of *in vitro* translation products by canine microsomal membranes. Transcripts from the bovine prolactin gene (BP), the spinach lipid transfer protein gene (LTP) and the chlorophyll a/b binding protein (LHCP) were translated without addition of microsomes (A,D,G), with microsomes (B,E,H) and with microsomes added after translation had been terminated (C,F). The numbers at the left indicate the apparent molecular weight of standards (in kDa).

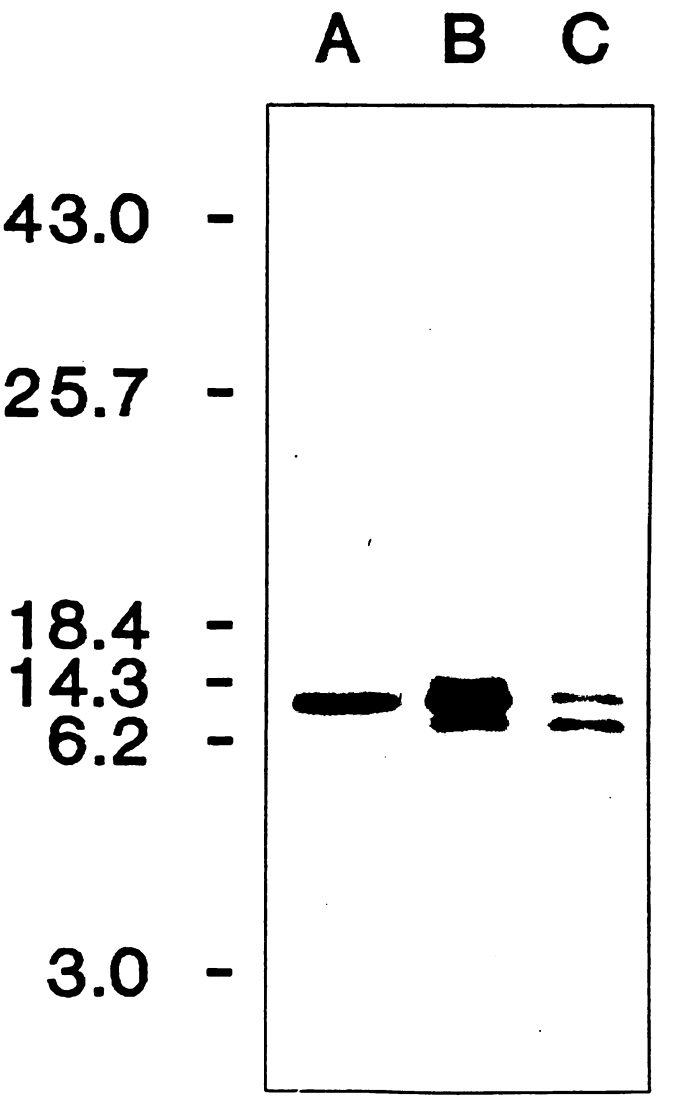

Figure 2-5. Processing of *in vitro* translation products by microsomal membranes from maize endosperm cell cultures. Transcripts of the lipid transfer protein were translated without the addition of microsomes (A), with maize microsomes (B), and with canine microsomes (C). The numbers at the left indicate the apparent molecular weight of standards (in kDa).

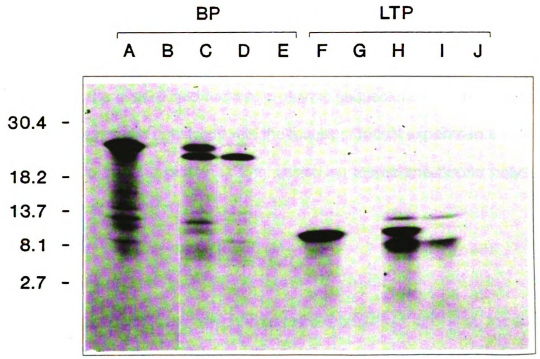

Figure 2-6. Protease sensitivity of translation products. Transcripts from the bovine prolactin gene (BP) and the spinach lipid transfer protein gene (LTP) were translated without addition of microsomes (A,B,F,G) or with dog microsomes (C,D,E,H,I,J). After translation, the reaction products were incubated with proteinase K for 1 h (B,D,E,G,I,J). In lanes E and J, Triton X-100 was added along with the proteinase K.

the high molecular weight product was not (Fig. 2-6D,I). The presence of microsomes also resulted in the appearance of a translation product from the lipid transfer gene of higher molecular weight. This does not appear to be due to N-linked glycosylation since the polypeptide does not contain the glycosylation sites Asn-X-Ser or Asn-X-Thr. Addition of low amounts of Triton-X100 to these translation mixtures concomitantly with addition of the proteinase K rendered the low molecular weight products protease sensitive (Fig. 2-6E,J). Thus, the evidence indicates that the mature lipid transfer protein is sequestered in the endoplasmic reticulum. This result is consistent with the results of recent experiments indicating that maize lipid transfer protein is synthesized on membrane-bound polysomes (Vergnolle, *et al.*, 1988).

DISCUSSION

The mechanisms by which lipids are transferred between intracellular membranes are not established (Bishop and Bell, 1988). However, the demonstrations that lipid transfer proteins could mediate rapid net transfer of lipid between a donor and acceptor membrane *in vitro* (Miguel, *et al.*, 1987) suggests that this mechanism could be important *in vivo*. According to this hypothesis, the rate of transfer of lipid between membranes would be proportional to the rate of diffusion of the protein-lipid complex between donor and acceptor membranes. In this respect, the high abundance of the spinach leaf protein (i.e., up to 4% of total soluble protein) in rapidly expanding tissue is consistent with such a

proposed role. By contrast, the very low abundance of the transcript in root tissue suggests that the protein is not involved in lipid transport in this tissue. This could, in principle, be due to the involvement of different isozymes in this tissue. Lipid transfer in leaves and other green tissues involves substantial flux between endoplasmic reticulum and chloroplast membranes (Browse, *et al.*, 1986). By contrast, in roots, relatively little flux of lipid is thought to take place between plastids and endoplasmic reticulum. Thus, lipid transfer proteins with different characteristics may be utilized in green and non-green tissues. The possibility of additional isozymes of related proteins is not excluded by the Southern analysis reported here since the conditions used would not have revealed homology to any DNA sequence of less than about 80% sequence identity. Because of the relatively large size of the spinach genome, and the fact that it has not yet been possible to genetically transform spinach, a detailed analysis of a weakly homologous gene family in this organism is not considered worthwhile. However, we have used the spinach cDNA clone described here to isolate the homologous cDNA and genomic clones from *Arabidopsis thaliana* (results not presented). The greatly reduced genome size of this organism, and the ease with which it can be genetically transformed, should facilitate a critical analysis of the question of tissue-specific isoforms.

The evidence, presented here, indicating that synthesis of the spinach lipid transfer protein involves cotranslational insertion into the endoplasmic reticulum, suggests that either this protein is synthesized by a novel mechanism, or it does

not participate in intracellular lipid transfer. Since it lacks the carboxy-terminal KDEL sequence associated with proteins retained in the lumen of the endoplasmic reticulum (Munro and Pelham, 1987) it is presumably transferred to another compartment of the cell by additional post-translational processing. Because the mature protein is normally recovered as a soluble extrachloroplastic protein, it has been assumed to be located in the cytosol (Kader, 1985). However, if the mature protein is primarily located in the cytosol, it would appear to be the first instance in which a cytosolic protein is synthesized via the secretory pathway with cleavage of the signal peptide. The only reported instance in which synthesis of a cytosolic protein involves cleavage of a signal sequence by signal peptidase is in the case of the hepatitis B virus pre-core protein (Garcia, *et al.*, 1987). However, in this case, translocation of the protein into the endoplasmic reticulum during synthesis is aborted at an early stage so that the protein is not inserted into the membrane. By contrast, the protease protection experiments indicate that the lipid transfer protein is taken up by the endoplasmic reticulum. There is no known mechanism by which it could subsequently be transfered to the cytoplasm.

If the spinach lipid transfer protein is not located in the cytosol, it cannot carry out the proposed role in transferring lipid between the endoplasmic reticulum and the other organelles. Unfortunately, the precise intracellular location of the protein has not been critically addressed by previous studies. Thus, a resolution of the alternate possibilities must await the results of an immunoelectron microscopic analysis of the cellular location of the protein.

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CHAPTER 3

AN *ARABIDOPSIS* NON-SPECIFIC LIPID TRANSFER PROTEIN IS A CELL WALL PROTEIN

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ABSTRACT

Lipid transfer proteins (LTPs), mediate the transfer of phospholipids between membranes *in vitro*. However, the *in vivo* function of LTPs is not known. To determine the precise location of the non-specific LTP from *Arabidopsis*, a cDNA clone was used to produce an *Arabidopsis* LTP:protein A fusion. Antibodies raised against the fusion protein were used to localize the *Arabidopsis* LTP by immunoelectron microscopy. LTP was found to be located in the cell wall, mainly in epidermal cells. This location appears to be inconsistent with the proposed role of the protein in intracellular lipid transfer, but is consistent with a possible function in cuticle formation.

INTRODUCTION

The mechanism by which lipids move from their main sites of synthesis, the endoplasmic reticulum (ER) and the chloroplast, to other cellular organelles is unknown. The search for proteins capable of intracellular lipid transfer resulted in the isolation of several small proteins which had the ability to mediate the *in vitro* transfer of radiolabeled phospholipids from liposomal donor membranes to mitochondrial and chloroplast acceptor membranes (Douday *et al.*, 1982, Kader *et al.*, 1984). It was, therefore, suggested that these proteins might catalyze lipid transfer between membranes within a cell (Arondel and Kader, 1990). However, the turnover number for the *in vitro* activity is very low and there is no evidence that these proteins carry out this role *in vivo*.

Lipid transfer proteins (LTPs) have been isolated from fungi, bacteria, plants, and mammals. Both specific and nonspecific LTPs (nsLTPs) have been isolated from mammalian tissue (Wirtz and Gadella, 1990), but only nsLTPs have been found in plant tissue (Arondel and Kader, 1990). Non specific LTPs have been isolated from maize (Douday *et al.*, 1982), spinach (Kader *et al.*, 1984), barley (Mundy and Rogers, 1986), and castor (Takishima *et al.*, 1986). Plant nsLTPs, while lacking any obvious sequence homology to animal LTPs, share high amino acid sequence similarity to the nsLTPs from other plant species. All known plant LTPs have several common characteristics; they are soluble, basic proteins with a molecular mass of approximately 9 kDa (Arondel, *et al.*, 1989) and, except for one cysteine to glutamine conversion in the ragi protein, all plant LTPs have 8

conserved cysteine residues (Bernhard and Somerville, 1989).

Based on the proposed function of intracellular lipid transfer, it has been assumed that LTPs were cytosolic (Arondel and Kader, 1990). However, recent evidence suggests that LTPs are not located in the cytosol. It has been reported that the maize LTP has an amino terminal extension and is synthesized on membrane bound polysomes (Tchang, *et al.*, 1988, Vergnolle, *et al.*, 1988) and that spinach LTP contains a signal peptide and is cotranslationally inserted into microsomal membranes (Bernhard, *et al.*, 1991). Since these proteins lack the endoplasmic reticulum retention signal, KDEL, they would be expected to be secreted or targeted to a specific organelle. In support of these expectations, Mundy and Rogers (1986) reported that a protein, which was subsequently identified as a barley LTP, was found in aleurone cell culture media. Similarly, Sterk, *et al.* (1991), recently demonstrated that a carrot LTP was secreted by carrot embryogenic cell cultures.

The expression pattern of LTP has been studied in cultured embryogenic carrot cells and in developing maize (Sossountzov, *et al.*, 1991; Sterk, *et al.*, 1991). *In situ* hybridization studies show that the carrot LTP mRNA is localized to the protoderm cells of somatic and zygotic embryos and in suspensor cells and the integument of zygotic embryos. The maize LTP RNA is expressed in the epidermis of the coleoptile and the scutellum. The localization of maize LTP has been investigated using immunogold labeling and light microscopy (Sossountzov, *et al.*, 1991). The highest concentrations of LTP were found in the outer epidermis of

coleoptiles and in the vascular tissue of the leaf.

To date, the localization of plant nsLTP in the aerial parts of mature plants has not been determined and the precise localization of LTPs has not been determined using electron microscopy. Therefore, I have used antibodies against an *Arabidopsis* LTP:protein A fusion for immunocytochemical labeling of various *Arabidopsis* tissues at the ultrastructural level. I show here that *Arabidopsis* LTP is located mainly in epidermal cell walls. The location of LTP outside of the plasma membrane and the preferential expression in epidermal cells are consistent with a previous proposal that LTP may participate in cuticle formation (Sterk, *et al.*, 1991).

EXPERIMENTAL PROCEDURES

Plant growth conditions - Columbia wild type of *Arabidopsis thaliana* (L.) Heynh. was used in all experiments. Plants were grown on a perlite:vermiculite:sphagnum mixture (1:1:1), at 22°C, under continuous illumination (100-150 μE $m^{-2}s^{-1}$).

Plasmid construction - The synthetic oligonucleotides, 5'TAAGGATCCAATACATCGAACGCT3'and5'AACTGCAGTACTCTGAAATTTCA3', were used as primers to generate a 510 bp PCR product from a previously isolated *Arabidopsis* LTP cDNA (Genbank accession M80566). This product was digested with EcoRV and BamHI and was ligated into the corresponding restriction

sites of pBluescript (Stratagene, San Diego, CA), to yield the plasmid pSLT1. The PstI and BamHI fragment from pSLT1 was ligated into pRIT2T (Pharmacia, Piscataway, NJ), a vector which contains the affinity tail of staphylococcal protein A, to yield the plasmid pSLT2, which was subsequently used to produce a LTP:protein A fusion protein. The protein A-LTP junction was sequenced to assure that these fragments were in the correct reading frame. pSLT3, which was used to produce an LTP:maltose binding protein fusion, was prepared by ligating the BamHI and PstI fragment of pSLT1 into the corresponding site of pIH821 (New England Biolabs, Beverly, MA), a plasmid which contains *malE*, the *E. coli* maltose binding protein. Restriction digests and ligation reactions were carried out as described by Maniatis et al. (1982).

Protein expression and purification - For production of the LTP:protein A fusion protein, *E. coli* strain N4830-1 (Pharmacia, Piscataway, NJ) containing pSLT2 was grown in 500 ml L-broth containing 50 mg/L ampicillin at 37°C until late log phase. At this point, the incubation temperature was rapidly increased to 42°C for 2 hours to induce expression of the fusion protein. Bacteria were collected by centrifugation, resuspended in 30 ml TST (50 mM Tris-Cl, pH 7.6, 150 mM NaCl, 0.05% Tween 20) and lysed using a French press. The lysed cells were centrifuged at 10,000 x g for 10 minutes and the supernatant was applied to an IgG affinity column (4 X 1.5 cm, Pharmacia, Piscataway, NJ). The fusion protein was eluted using 0.5 M HOAc, adjusted to pH 3.4 with NH_4OAc. The eluent was

lyophilized and resuspended in 25 mM sodium phosphate buffer, pH 7.5. The sample was then applied to a MonoQ column (5.5 X 0.7 cm) and eluted with a 50 ml linear gradient of 0 to 1 M NaCl in 40 mM Tris-Cl, pH 8.0. The purity of the protein was monitored by SDS-PAGE (Laemmli, 1970).

For production of the LTP:MalE fusion protein, *E. coli* strain DH5α containing pSLT3 was grown in 1 liter L-broth containing 50 mg/L ampicillin to mid log phase. At this point, IPTG was added to a final concentration of 0.3 mM. After 2 hours, cells were collected by centrifugation and resuspended in 40 ml 10 mM sodium phosphate, 30 mM NaCl, 0.25% Tween 20, 10 mM β-mercaptoethanol, 10 mM EDTA, pH 7. Cells were lysed using a French press, centrifuged at 9000 x g for 30 min and the supernatant applied to an amylose column (13 X 2.5 cm). The amylose column was prepared adding 60 ml 5 N NaOH and 30 ml epichlorhydrin to 40 ml 25% (w/v) amylose. The resulting gel was fragmented in a Waring blender for approximately 5 seconds. The protein was eluted from the amylose column with 3 ml 10 mM $NaPO_4$, 0.5 M NaCl, 10 mM β-mercaptoethanol, 10 mM maltose (pH 7.0).

Antibody production - Approximately 50 ug of the purified fusion protein was suspended in Freund's complete adjuvant and subcutaneously injected into a New Zealand white rabbit. Three and five weeks after the initial injection, the rabbit was reinjected with 25 ug of the protein suspended in Freund's incomplete adjuvant. The rabbit was bled at two week intervals after the final injection for a total of 10

weeks.

Approximately 30 mg of purified LTP-MalE fusion protein was to bound to 3 ml Affigel 15 (Bio Rad, Richmond, CA) according to the manufacturer's instructions. Fifteen ml crude antiserum was added to the affinity column, the column was washed with several volumes of 25 mM NaH_2PO_4 pH 7.4, and the anti LTP antibody was eluted with 3 ml 200 mM glycine pH 2.8. The specificity of the affinity purified antibody was checked by Western analysis.

Protein isolation and protein gel blot analysis - An *Arabidopsis* protein extract was prepared by homogenizing tissue in 20 mM glycine pH 8.4, 5 mM $MgCl_2$, 2.5 mM EDTA. Extracts were centrifuged twice at 10,000 X g for 10 min. Protein concentration was determined by the BCA protein assay (Pierce Biochem, Rockford, IL). Forty ug of protein was electrophoretically resolved on a 10-20% gradient SDS-polyacrylamide gel. Proteins were transferred to nitrocellulose and Western analysis was carried out essentially as described by Towbin, *et al.* (1979). Goat anti-rabbit IgG-alkaline phosphatase conjugate was used as the second antibody. Alkaline phosphatase activity was revealed using nitroblue tetrazolium and 5-bromo-4-chloro-3 indolyl phosphate as substrates.

Fixation of tissue - Plant tissue was fixed for 1.5 h at room temperature in 2% (v/v) glutaraldehyde in 50 mM potassium phosphate, pH 7.0. The samples were washed three times with phosphate buffer and post-fixed for 1 h in 1% OsO_4

in 50 mM potassium phosphate buffer, pH 7.0 at room temperature. The tissue was washed three times in phosphate buffer, dehydrated in a graded series of acetone (10-95%), and infiltrated with LR White resin (Polysciences, Warrington, PA). Infiltration was carried out over 36 h with several changes of resin. Dehydration and infiltration were carried out at 4°C. The resin was polymerized for 24 h at 55°C, in 1 ml gelatin capsules.

Immunogold labeling for electron microscopy - Ultrathin sections (gold, approximately 80-100 nm, on average 40 sections of each of 5 sample prepartions of each tissue type) of various tissues were mounted on 300 mesh nickel grids. The sections on grids were treated with 5% $NaIO_4$ for 30 min and 0.1 N HCl for 10 min. After a 5 min wash in distilled water, the grids were incubated in TBST (10 mM Tris, 150 mM NaCl, 0.05% Tween 20, 0.02%NaN_3, adjusted to pH 7.5 with HCl) containing 1% BSA for 20 min. The sections were labeled overnight with a 1:20 dilution of the primary antibody in TBST-BSA. After labeling, the grids were washed 3 times with TBST and then treated with a 1:50 dilution of 15 nm colloidal gold-conjugated protein A (EY Labs, San Mateo, CA) for 45 min. The grids were washed three times in TBST, treated with 1% glutaraldehyde in 0.05 M potassium phosphate buffer for 5 minutes and rinsed in distilled water. The sections were stained for 30 min with a saturated uranyl acetate solution and 5 min with Reynold's lead citrate. The sections were viewed and micrographs were taken with a Philips 201 electron microscope (Philips Netherlands, Eindhoven).

Immunocytochemical controls - The specificity of labeling was tested by (1) labeling with preimmune serum in place of the anti-LTP primary antibody; (2) incubating sections with secondary antibody without prior exposure to primary antibody; (3) incubating antibody with maltose binding protein:LTP fusion protein before labeling; (4) treating sections made from transgenic plants in which the presence of LTP was reduced by the expression of an antisense construct of the LTP cDNA.

RESULTS

Protein expression and antibody production - In order to produce anti-LTP antibodies of suitable quality for immunoelectron microscopy, two different LTP-fusion proteins were constructed which permitted facile purification of the LTP by affinity chromatography. A PCR product corresponding to the complete open reading frame of the mature *Arabidopsis* LTP was subcloned into the plasmid pRIT2T, to yield the plasmid pSLT2 (Fig. 3-1A). This plasmid, which contains the LTP open reading frame fused to the protein A affinity tail, permits purification of the fusion protein on an IgG column. The same PCR product was subcloned into the plasmid pIH821, to yield pSLT3 (Fig. 3-1B). This plasmid, which contains the LTP open reading frame fused to the *E. coli* maltose binding protein, permits affinity purification of the fusion protein on an amylose column.

Expression of an *Arabidopsis* LTP:protein A fusion in *E. coli* facilitated purification of a fusion protein which was uncontaminated by other plant proteins

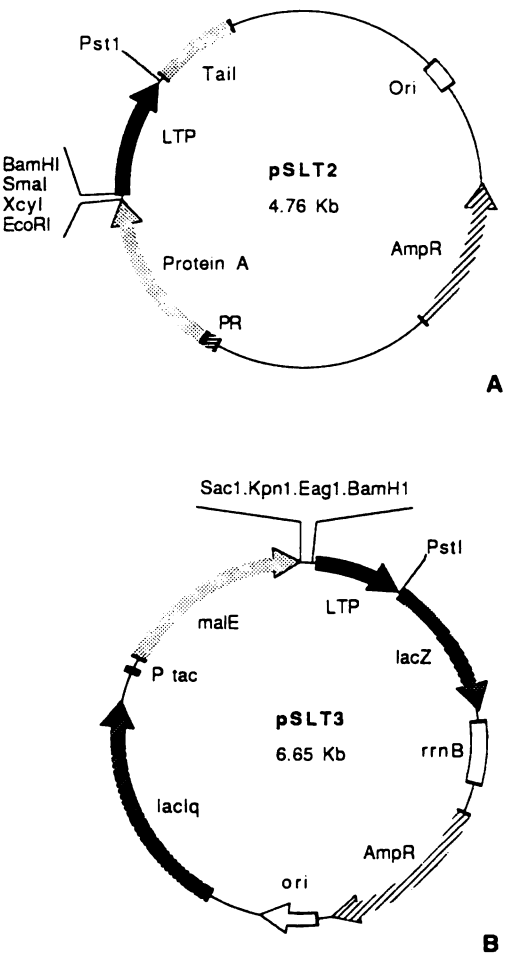


Figure 3-1. Structure of plasmids used to produce LTP fusion proteins.

A) The LTP open reading frame is fused to the affinity tail of staphylococcal protein A. Abbreviations: Tail, 3'end of protein A gene; AmpR, ampicillin resistance; ori, origin of replication.

B) The LTP open reading frame is fused to *malE*, the *E. coli* maltose binding protein. Abbreviations: $laqI^q$, hyper-expressed *lac* repressor; P tac, tac promoter; malE, maltose binding protein; lacZ, β-galactosidase; AmpR, ampicillin resistance; ori, origin of replication.

(Fig. 3-2). Western analysis using antibodies raised against the purified LTP:protein A fusion showed that the antibody recognized a protein in crude *Arabidopsis* leaf extracts with a molecular weight of about 9 kDa, which is the size expected for the mature LTP. However, there was weak cross reaction with several other proteins (data not shown). To ensure that the anti-LTP antibodies would cross react only with LTP during immunolocalization, the antibodies were purified by affinity chromatography on an LTP:maltose binding protein fusion coupled to Affigel 15. The resulting antibodies recognized a single 9 kDa protein in Western blots of total protein extracts from *Arabidopsis* leaves (Fig. 3-3). No immunostaining was observed with the preimmune control serum (Fig. 3-3).

Arabidopsis LTP is found in many tissues - Protein extracts were made from a variety of tissue types, including root, leaf, petiole, stem, silique, and flower. Western analysis showed that LTP was present in all tissue types examined (Fig. 3-4). Expressed as a percentage of total protein, the LTP level in the stem was the highest of all tissue types examined. The LTP level in flowers was higher than that in leaves, petioles, siliques, and roots. LTP was detected in root tissue, although at much lower levels than in other plant tissue (Fig. 3-4).

Arabidopsis LTP has an extracellular location - Immunogold labeling at the ultrastructural level was used to determine the specific cellular location of LTP. Examination of sections from leaf, petiole, stem, root, and flower showed that the

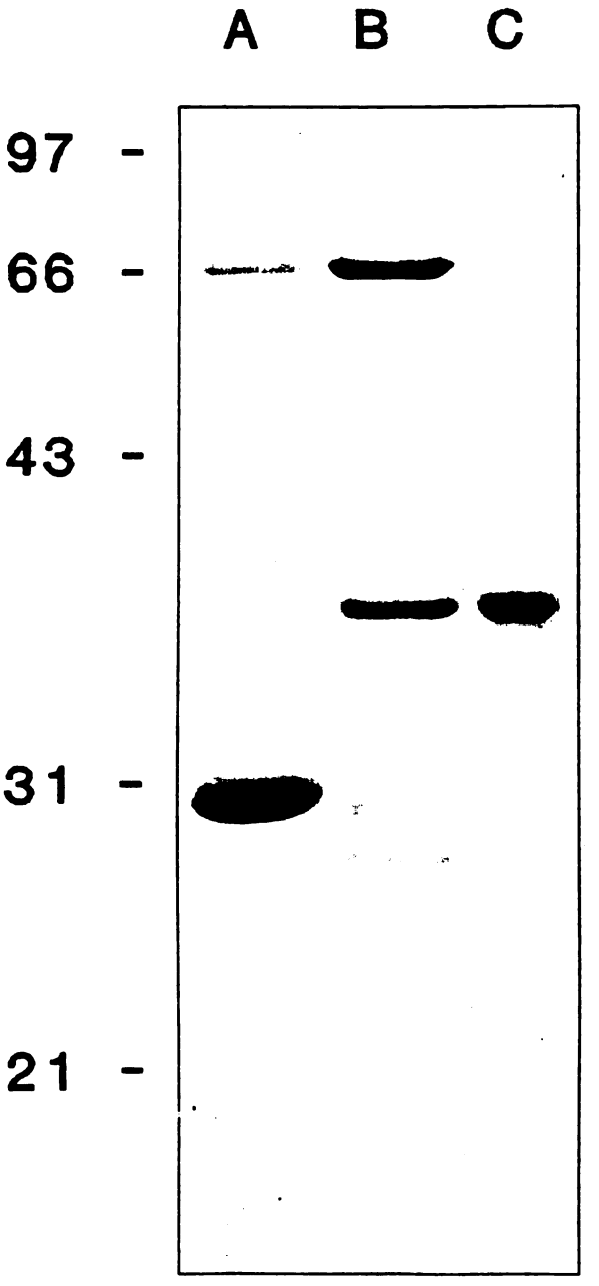


Figure 3-2. SDS-PAGE of purified LTP-protein A fusion from *E. coli.*

Proteins were expressed in *E. coli*, purified by IgG affinity chromatography, and resolved by SDS-PAGE. Lane A) Protein A; Lane B) LTP-protein A fusion; Lane C) LTP-protein A fusion after further purification by anion exchange chromatography.

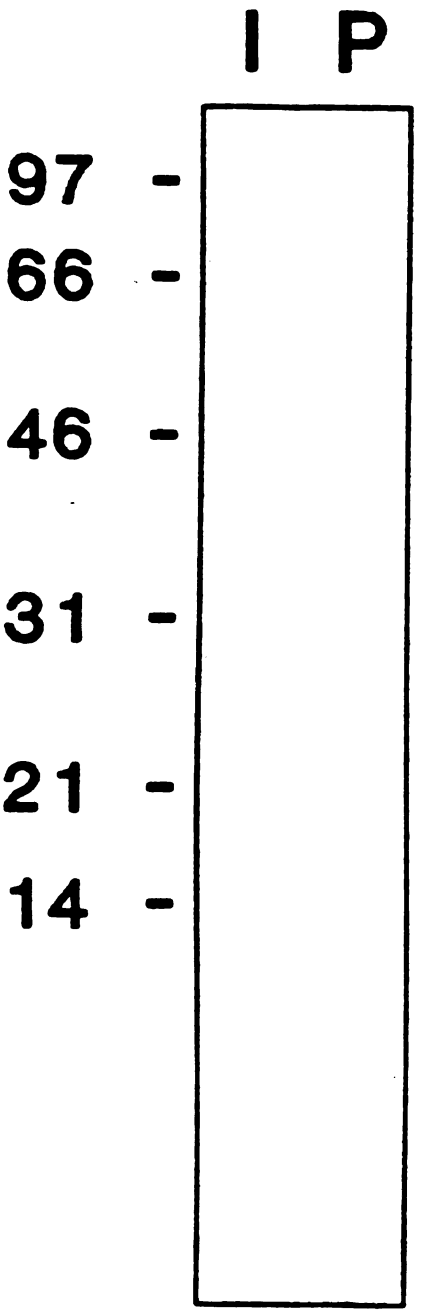


Figure 3-3. Western blot analysis of an *Arabidopsis* leaf protein extract produced with anti-LTP:protein A antibodies.

An *Arabidopsis* leaf protein extract (40 μg per lane) was resolved by SDS-PAGE, transferred to nitrocellulose, and immunodetected with affinity purified anti-LTP antibodies (Lane I) or preimmune serum (P).

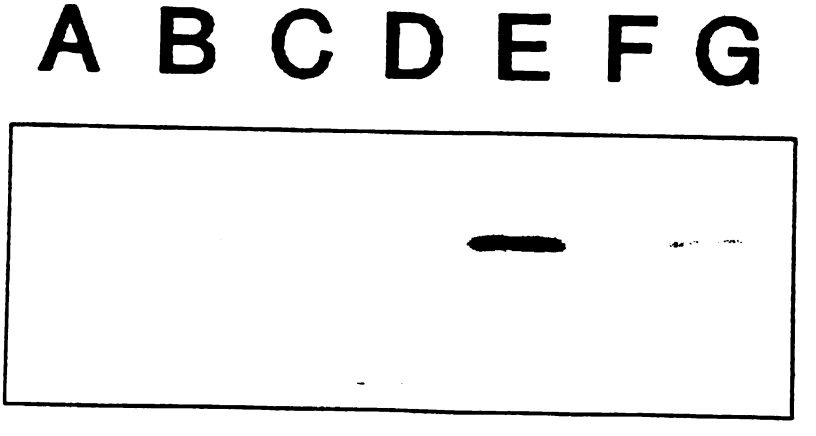


Figure 3-4. Tissue distribution of LTP.

Protein extracts (40 μg per lane) were prepared from various tissues of *Arabidopsis* plants, resolved by SDS-PAGE, transferred to nitrocellulose and immunodetected with anti-LTP antibodies. A) root; B) petiole; C) newly emerging leaf; D) fully expanded leaf; E) stem; F) silique; G) flower.

LTP was located outside of the plasma membrane in the cell wall (Figs. 3-5, 3-6, 3-7). The highest concentration of LTP was seen in the epidermal cell walls in the aerial parts of the plant. Figure 4 shows the immunolabeling of epidermal cell walls from sections through the tip of a mature leaf (Fig. 3-5A), a petiole (Fig. 3-5C), and a stem (Fig. 3-5E). The labeling in the leaf, petiole, and stem sections was confined to the cell wall and cuticle. There was essentially no labeling when preimmune serum is used in place of anti LTP antibodies (Fig. 3-5B, D, F), or when anti-LTP antibodies were quenched with an LTP:maltose binding protein fusion protein before labeling (data not shown). Thus, the labeling was specific for LTP.

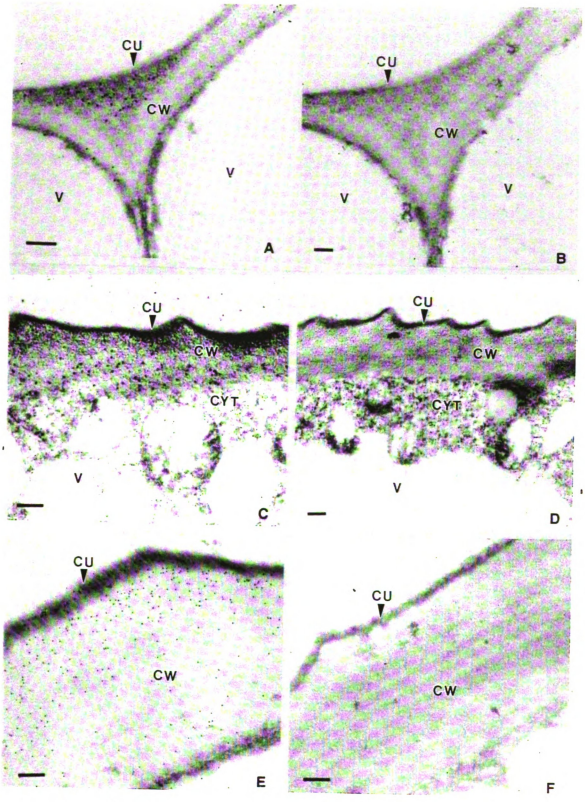
Examination of sections of leaf tissue indicated that the protein was found in equal amounts in both the upper and lower epidermis, and revealed that the walls of guard cells were much more heavily labeled than the walls of other leaf epidermal cells (Fig. 3-5A, 3-6A). Labeling was observed around the entire epidermal cell; the outer periclinal wall, the anticlinal wall, and the inner periclinal wall (data not shown). Labeling was also observed in cell walls of stem cortical cells (Fig. 3-6B), and petiole cortical cells (data not shown). Light labeling was also observed in the walls of leaf mesophyll cells. In stem cortical cells, labeling was more intense in the cell walls adjacent to intercellular space than in walls between two adjacent cortical cells (Fig. 3-7B). An unusual type of cellular material, possibly a secretory product, was seen in certain intercellular spaces of stem cortical cells. The exact nature of this material is unknown, but it was heavily labeled (data not shown). Labeling was observed in the secondary walls of

Figure 3-5. Immunocytochemical localization of LTP in the cell wall of *Arabidopsis* leaf tip, stem and petiole using immunogold labeling.

Anti-LTP antibodies label the cell wall of leaf, petiole and stem epidermal cells. On average, 40 sections of each of 5 sample preparations of each tissue type were examined for each treatment (using immune serum and preimmune serum). There is essentially no labeling when preimmune serum is used in place of anti-LTP serum.

A) Leaf tip, longitudinal section, treated with anti-LTP serum.
B) Leaf tip, longitudinal section, treated with preimmune serum.
C) Petiole epidermal cell wall, treated with anti-LTP serum.
D) Petiole epidermal cell wall, treated with preimmune serum.
E) Stem epidermal cell wall, treated with anti-LTP serum.
F) Stem epidermal cell wall, treated with preimmune serum.

A-F, Bar = 0.25 μm
cu, cuticle; cw, cell wall; cyt, cytoplasm; v, vacuole


CU
CW
V
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A
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V
B
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CW
CYT
V
C
CU
CW
CYT
V
D
CU
CW
E
CU
CW
F

Figure 3-6. Immunocytochemical localization of LTP in *Arabidopsis* guard cells, stem cortical cells, and xylem vessels using immunogold labeling.

Anti-LTP antibodies label the cell wall surrounding guard cells and stem cortical cells. On average, 40 sections of each of 5 sample preparations of each tissue type were examined for each treatment (using immune serum and preimmune serum).
A) Guard cells of leaf epidermis, longitudinal section, treated with anti-LTP serum. Gold particles are present in the cell wall surrounding these cells. Bar = 0.5 μm
B) Stem cortical cells, treated with anti-LTP serum. Gold particles are present in the cell walls, most notably of cells adjacent to intercellular space (arrowheads). Bar = 0.25 μm
C) Xylem vessel from stem, treated with anti-LTP serum. The secondary cell wall is labeled. Bar = 0.5 μm
D) Xylem vessel from stem, treated with preimmune serum. The secondary cell wall is labeled. Bar = 0.5 μm

cu, cuticle; cw, cell wall; cyt, cytoplasm; is, intercellular space; sw, secondary cell wall; v, vacuole; xp, xylem parenchyma

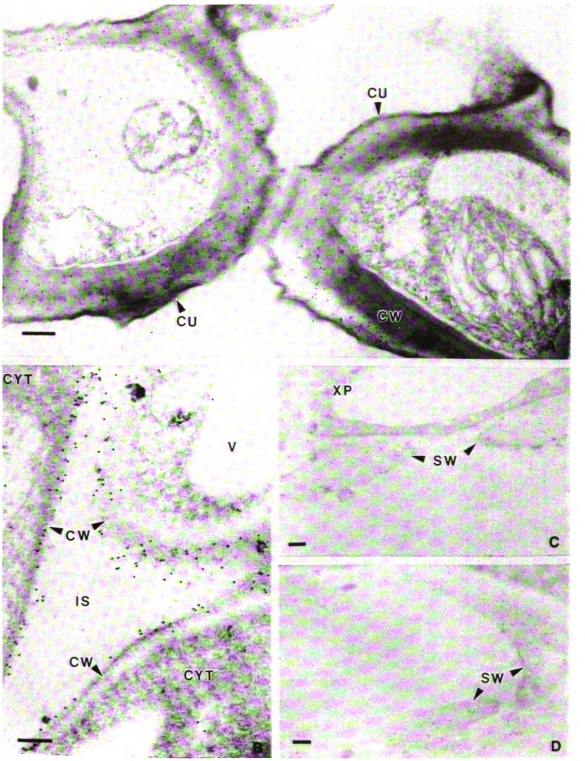
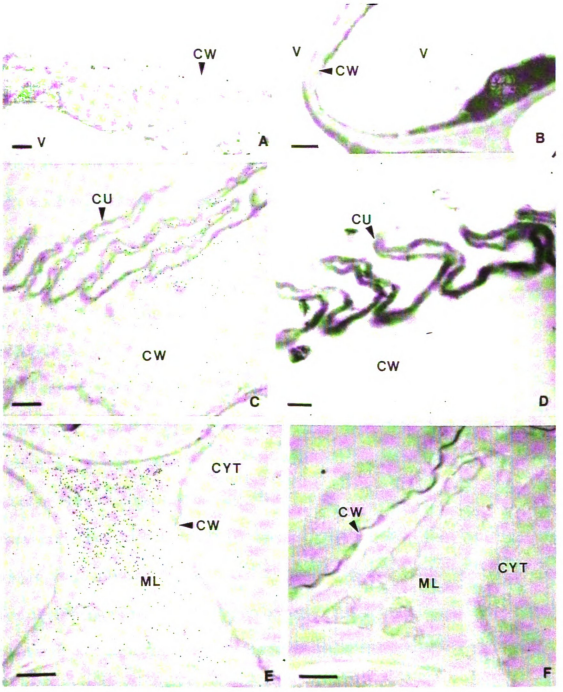

CU
CU
CW
CYT
V
CW
IS
CW
CYT
B
XP
SW
C
SW
D

Figure 3-7. Immunocytochemical localization of LTP in *Arabidopsis* root and pistil using immunogold labeling.

Anti-LTP antibodies label the root epidermal cell walls, the stigma papillae and cell wall at the stigma surface, and the intercellular space between the parenchymous cells directly below the stigma surface. On average, 40 sections of each of 5 sample preparations of each tissue type were examined for each treatment (using immune serum and preimmune serum).
A) Root epidermal cell wall, treated with anti-LTP serum. Gold particles are seen in the wall.
B) Two root endodermal cells, treated with anti-LTP serum. Note the absence of gold particles associated with these cells.
C) Stigma surface, longitudinal section, treated with anti-LTP serum. Gold particles are seen associated with the cell wall and stigma papillae.
D) Stigma surface, longitudinal section, treated with preimmune serum. Note the absense of gold particles.
E) Intercellular space of stigma, treated with anti-LTP serum. Notice the heavy labeling over this area.
F) Intercellular space of stigma, treated with preimmune serum. Note the absence of labeling in this area.

A-F, Bar = 0.25 μm
cu, cuticle; cw, cell wall; cyt, cytoplasm; ml, middle lamella; v, vacuole


CW
V
A
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CW
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CU
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C
CU
CW
D
CYT
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ML
E
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ML
CYT
F

vascular tissue. However, this labeling occurred when either anti-LTP antiserum or preimmune serum was used (Fig. 3-6C, D), indicating that the labeling was not specific for LTP. There was light labeling of root epidermal walls (Fig. 3-7A) but no labeling associated with endodermal cells (Fig. 3-7B) or with any other cell type in the root. LTP was present in the walls of the cells on the surface of the stigma (Fig. 3-7C) and in the cell walls of the stigma parenchyma. Fig. 3-7E shows the junction between four parenchymous cells just below the surface of the stigma in which labeling was associated with the middle lamella. Essentially no labeling was associated with the stigma surface or intercellular space when preimmune serum is used in place of anti-LTP antibodies (Fig. 3-7D, F).

DISCUSSION

Lipid transfer proteins were originally isolated based on their ability to catalyze lipid transfer between membranes *in vitro*. However, it has not been possible to assign an *in vivo* function to these proteins. A yeast phosphatidylinositol transfer protein (PI-TP) is the only LTP to which the *in vitro* activity correlates to a function *in vivo* (Bankaitis, *et al.*, 1990). This protein appears to be involved in elevating the phosphatidylinositol/phosphatidylcholine ratios of yeast Golgi membranes by an unknown mechanism (Cleves, *et al.*, 1991). However, the nsLTPs from plants lack sequence homology to the yeast PI-TP and thus, likely, have a different biological role.

Although it has long been assumed that LTPs are cytosolic (Helmkamp,

1986), there is no evidence to support such an assumption, and recent evidence indicates that plant LTPs are not located in the cytosol, but are secreted (Bernhard, *et al.*, 1990; Mundy and Rogers, 1986; Sterk, *et al.*, 1991). Using immunocytochemical techniques and light microscopy, Sossountzov, *et al.* (1991), demonstrated that a maize LTP is found at the periphery of leaf and coleoptile epidermal cells and suggested that the staining was associated with the cytoplasm. However, since light microscopic techniques lack the capability to resolve the plasma membrane, it is generally necessary to use electron microscopic imaging techniques to determine on which side of the plasma membrane a protein is located. In order to resolve this issue I have examined an extensive series of ultrathin sections from all major tissues of mature *Arabidopsis* plants by immunoelectron microscopy using highly purified monospecific antibodies. My results indicate that the LTP is primarily or solely located in the cell wall and cuticle of all cell types where it is represented. Although this technique cannot exclude the possibility of relatively low levels of accumulation in other cellular compartments, the weight of evidence appears to be inconsistent with a cytosolic location. In particular, since the *Arabidopsis* LTP contains a signal peptide and the similar proteins in spinach and barley enter the secretory pathway (Bernhard, *et al.*, 1991; Madrid, 1991), it was not unexpected to find this protein in the cell wall. This localization, however, would seem to preclude any direct involvement of this LTP in lipid transfer between organellar and subcellular membranes.

The *Arabidopsis* LTP was detected in the epidermal cell walls of all tissue types examined; leaves petioles, stems, flowers, and, to a minor extent, roots. The amount of labeling seen in the stem is higher than that seen in other green tissues examined. Guard cells were more heavily labeled than the surrounding epidermal cells. Although it has been reported that LTP is strongly expressed in vascular strands (Sossountzov, *et al.*,1991), I did not observe specific labeling of vascular tissue. Western analysis shows that the *Arabidopsis* LTP is present in low concentrations in roots, and immunocytochemical labeling shows that this protein can be detected in the cell walls of epidermal cells. LTP is present in the walls of cells on the stigma surface and is also associated with the walls of the parenchymous cells of the stigma. The epidermal and subepidermal layers of a stigma can produce a secretion that eventually form a film over the epidermal walls (Esau, 1977). The secretion contains mainly lipid and phenolic compounds, chiefly anthocyanins, flavonoids, and cinnamic acids (Martin and Brewbaker, 1971). LTP could, in principle, play a role in deposition of the lipid components of the secretion.

The preferential accumulation of LTP in the cell walls and cutin layer of epidermal cells is consistent with the results reported by Sterk, *et al.* (1991) and Sossountzov, *et al.* (1991), and is consistent with speculation that the protein might play a role in cuticle formation (Sterk, *et al*, 1991). The cuticle is a continuous layer of predominantly lipophilic material found on the outermost surface of the aerial parts of plants (Holloway, 1982). The major component of the cuticle is cutin, an

insoluble polymer composed mainly of C_{16} and C_{18} hydroxylated fatty acids (Holloway, 1982). The cuticle also contains wax, which is composed of a diverse group of long chain hydrocarbons, including long chain fatty acids esterified with alcohols, and free aldehydes, ketones, and alcohols (Kolattukudy, 1980). The aliphatic chains present in plant waxes and cutin are generated within the epidermal cell and are delivered to the outside of the cell where it is thought that esterification takes place (Croteau and Kolattukudy, 1974). It is possible that LTPs, which have a demonstrated ability to bind fatty acids (Rickers, *et al.*, 1985), could bind, and therefore solubilize, wax or cutin monomers upon release from the plasma membrane and deliver them to the developing cuticle where they would be available for polymerization. However, since it is not generally accepted that roots contain cutin, the presence of LTP in this tissue is inconsistent with a role for this protein in cuticle formation.

Another possible function of plant LTPs could be a general role in plant defense. A protein, which is homologous to nsLTPs, has recently been isolated from radish seeds and shows antifungal activity *in vitro* (W. Broekaert, personal communication). Also, thionins, which have toxic effects on bacteria, fungi, and insects (Bohlman and Apel, 1991), have several features in common with plant LTPs; they are low molecular weight, basic proteins with several conserved cysteine residues. A barley leaf thionin is found in the cell wall, with the highest concentration in the outer wall of the epidermal cell layer (Bohlman, *et al.*, 1988; Reimann-Philip, *et al.*, 1989). This localization is similar to that of *Arabidopsis* LTP

in all green tissue examined.

A complication concerning the possible role of LTP is that in some plant species there are isoforms which might have different functions. Four isoforms of LTP isolated from castor bean seedlings are differentially expressed in a tissue-specific manner (Tsuboi, *et al.*, 1991). In a preliminary report, Yamada and coworkers (1990) have reported that in castor bean cotyledons, the LTP-A isoform is present in glyoxosomes and in the secondary wall of xylem vessels. This observation is very surprising since different mechanisms are normally required to target proteins to these cellular compartments. Two different LTPs have been found in tobacco, one of which is present predominantly in tapetal cells (Koltunow, *et al.*, 1990). A second tobacco LTP gene is expressed in all aerial portions of the plant (Fleming, *et al.*, 1992). A second gene was detected in *Arabidopsis* by probing Southern blots at high stringency with the LTP cDNA (chapter 4 of this dissertation). A second LTP from *Arabidopsis* has recently been isolated (F. Garcia-Olmeda, personal communication), and it is possible that this isoform has a differing localization than that described in this chapter. The anti-LTP antibodies which are described in this chapter will be tested for cross reaction with the recently isolated isoform.

In conclusion, due to its extracytoplasmic location, the nsLTP from *Arabidopsis* is unlikely to be directly involved in intracellular lipid transfer. Thus, assuming that the lipid transfer activity is not incidental to the function of the protein, it is possible that this protein is involved in cuticle formation or in some

aspect of general plant defense. Analysis of transgenic plants in which the amount of nsLTP has been greatly reduced by expression of an antisense construct of the LTP cDNA (S. Thoma, U. Hecht, and C. Somerville, unpublished results) may facilitate the elucidation of an *in vivo* function for plant nsLTPs.

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CHAPTER 4

AN *ARABIDOPSIS* LIPID TRANSFER PROTEIN PROMOTER SPECIFIES COMPLEX EXPRESSION IN TRANSGENIC PLANTS

ABSTRACT

Lipid transfer proteins (LTPs) are a class of proteins which have the ability to stimulate phospholipid transfer between membranes *in vitro*. Based on their extracellular location, however, it is unlikely that these proteins have a similar role *in vivo*. To better understand a possible role for these proteins, an LTP gene from *Arabidopsis* has been cloned. The regulation of the LTP gene was examined by analysis of GUS activity in transgenic plants containing LTP promoter-ß-glucuronidase fusions. The LTP promoter was active early in seedling development and was strongly expressed in the tips of cotyledons, in the shoot meristem, in stipules, and at the base of lateral roots. In adult plants, the gene fusion was expressed in leaf guard cells, stem epidermal cells, and the stigma, pollen grains and floral nectaries of mature flowers. Analysis of the promoter region shows the presence of sequences homologous to putative regulatory elements of several genes encoding proteins of the phenylpropanoid biosynthetic pathway. These sequence data, along with the GUS expression pattern are consistent with a role for the *Arabidopsis* nsLTP in phenylpropanoid metabolism or regulation of expression by the same or similar mechanisms.

INTRODUCTION

The mechanisms by which lipids move from their sites of synthesis, the endoplasmic reticulum (ER) and the chloroplast, to other cellular organelles, is unknown. A class of proteins, called lipid transfer proteins (LTPs) was first isolated based on the ability to transfer phospholipids between natural and artificial membranes *in vitro* (Wirtz and Zilversmit, 1968). It has been difficult to assign an *in vivo* function to these proteins, but it has long been assumed that they are involved in intracellular lipid transport (Helmkamp, 1990).

LTPs have been isolated from mammalian tissue (Kamp, *et al.*, 1973; Helmkamp, *et al.*, 1974; for review see Wirtz, 1982), bacteria (Tai and Kaplan, 1985), yeast (Szolderits, *et al.*, 1989; Paltauf and Daum, 1992), filamentous fungi (Tan, *et al.*, 1990), and several plant species, including spinach (Kader, *et al.*, 1984, Bouillon, *et al.*, 1987), maize (Douady, *et al.*, 1982), barley (Mundy and Rogers, 1986), castor (Takishima, *et al.*, 1986), and sunflower (Arondel, *et al.*, 1990). The plant LTPs are all small, soluble, basic proteins. Unlike some of the mammalian and yeast proteins, which transfer only a specific class of phospholipids, plant LTPs have a broad specificity for the type of lipid they will transfer, and the spinach and castor LTPs have a demonstrated ability to bind fatty acids (Rickers, *et al.*, 1985; Yamada, *et al.*, 1990).

Based on their proposed function of intracellular lipid transfer, it has been presumed that LTPs were cytosolic proteins (Arondel and Kader, 1990; Helmkamp, 1990). However, recent evidence demonstrates that plant LTPs are not located in

the cytoplasm. It has been reported that the maize LTP has an amino terminal extension and is synthesized on membrane bound polysomes (Tchang, *et al.*, 1988) and the spinach and barley LTPs contain signal peptides and are cotranslationally inserted into the ER (Bernhard, *et al.*, 1991; chapter 2 of this dissertation; Madrid, 1991). Since these proteins lack the ER retention signal, H/KDEL (Munro and Pelham, 1987), they would be expected to be secreted or targeted to a specific organelle. In support of these expectations, Mundy and Rogers (1986) reported that a barley protein, which was subsequently identified as an LTP (Bernhard and Somerville, 1989; Breu, *et al.*, 1989) was found in the aleurone cell culture medium, and Sterk, et al., (1991) demonstrated that a carrot LTP was secreted by embryogenic cell cultures. Sossountzov, et al. (1991) have shown that a maize LTP is localized to the periphery of the epidermal cells in maize coleoptiles, and suggest that the labeling is associated with the cytoplasmic side of the plasma membrane. It has more recently been demonstrated, using immunocytochemical labeling at the ultrastructural level, that an *Arabidopsis* LTP is localized to the cell wall, and that it is mainly seen in epidermal cell walls (Thoma, *et al.*, 1993; chapter 3 of this dissertation).

With the mounting evidence that at least some plant nsLTPs are extracellular, the proposed function of these proteins in intracellular lipid transfer comes into question. Based on the observation that a carrot LTP is secreted and the localization of an *Arabidopsis* LTP to the walls of epidermal cells, it has been proposed that the protein may play a role in cuticle formation or in general plant

defense (Sterk, *et al.*, 1991; Thoma, *et al.*, 1993; chapter 3 of this dissertation). However, analysis of transgenic plants which contain a reduced level of LTP due to the presence of an *Arabidopsis* LTP cDNA in reverse orientation behind the 35S promoter (chapter 5 of this dissertation), shows no alteration in wax or cutin composition under normal growth conditions. To better understand this protein and its possible function, a genomic clone corresponding to an *Arabidopsis* LTP has been isolated. I show here that LTP is encoded by at least two genes in *Arabidopsis* and have designated this clone ALTP1. A series of transgenic plants has been produced which contains the LTP promoter fused to the ß-glucuronidase reporter gene. I have analyzed the temporal and spatial expression of this promoter and have found it to be expressed most strongly in the stigma surface, epidermal cells of the leaf and stem, floral nectaries, and pollen grains of mature plants. The promoter was also active in early seedling development and was strongly expressed in the tips of cotyledons, in the shoot meristem, in stipules, and at the base of lateral roots. The pattern of expression suggested a role for this protein in phenylpropanoid metabolism. Computer aided analysis of the promoter region shows that it contains several putative regulatory sequences found in promoters of biosynthetic genes for the phenylpropanoid pathway (Cramer, *et al.*, 1989; Lois, *et al.*, 1989; Ohl, *et al.*, 1990).

EXPERIMENTAL PROCEDURES

Plant Material - Arabidopsis thaliana ecotype RLD was used to produce all transgenic plants as it had the best transformation efficiency of all ecotypes tested (J. Schiefelbein, unpublished results). Transgenic seed were surface sterilized with a solution containing 5% hypochlorite and 0.02% Triton X-100, rinsed several times with sterile water, and plated on Murashige-Skoog (MS) medium (Murashige and Skoog, 1962) containing kanamycin (50 μg/ml). Plants were grown at 22°C under continuous illumination (100-150 $\mu E\ m^{-2}s^{-1}$). With the exception of plants used for GUS expression during seedling development, kanamycin resistant seedlings were transplanted to pots containing a mixture of perlite:vermiculite:sphagnum (1:1:1) and grown under the same light and temperature conditions.

Isolation of an Arabidopsis LTP genomic clone - A λGEM11 *Arabidopsis* (ecotype Columbia) leaf genomic DNA library was plated on *Escherichia coli* KW51 cells and resulting plaques were lifted onto nitrocellulose filters. The filters were prehybridized for 15 min at 65°C in a solution containing 200 mM NaCl, 20 mM sodium phosphate, pH 7.7, 2 mM EDTA, 1% SDS, 0.5% dry milk powder, 10% dextran sulfate. Hybridization was carried out for 8 h at 65°C in the same solution containing an *Arabidopsis* LTP cDNA which was labeled with [α-^{32}P] dCTP by random priming (Feinberg and Vogelstein, 1983). The filters were washed three times for 10 min at 65°C in a solution containing 2X SSC, 0.1% SDS and once in a solution containing 0.5X SSC. 0.5% SDS under the same conditions. A 1.9 kb

EcoRI fragment containing the complete coding sequence for the *Arabidopsis* LTP1 gene and over 1 kb of the 5' upstream region was subcloned into the corresponding site of pBluescript (Stratagene, La Jolla, CA). Single stranded DNA was sequenced by the dideoxy chain-termination method (Sanger, *et al.*, 1977). All nucleic acid manipulations were carried out as described in Maniatis, *et al.* (1982).

Nucleic acid manipulations - DNA was extracted from leaves of three week old *Arabidopsis* by grinding 10 g tissue in liquid N_2 and adding to 10 ml of a 65°C solution containing 2% (w/v) hexadiacyltrimethyl ammonium bromide (CTAB), 100 mM Tris-Cl, pH 8, 20 mM EDTA, 1.4 M NaCl, 1% polyvinylpyrrolidone. The mixture was extracted once with chloroform:isoamyl alcohol (24:1). The aqueous phase (10 ml) was added to 1 ml 10% CTAB, 0.7 M NaCl and this was extracted again with chloroform:isoamyl alcohol. Ten ml of a solution containing 1% CTAB, 50 mM Tris-Cl, pH 8, and 10mM EDTA was added to the aqueous phase and the mixture was centrifuged at 10,000 x g for 10 min. The pellet was suspended in 2 ml high salt TE (10 mM tris-Cl, pH 8, 1 mM EDTA, 1 M NaCl). The DNA was recovered by ethanol precipitation and finally resuspended in TE.

For Southern analysis, DNA was digested and resolved by electrophoresis through 0.8% agarose (1.7 μg/lane). The gel was then treated for 15 min with 0.25 N HCl, followed by 30 min in denaturation solution (0.5 M NaOH, 1.5 M NaCl) and 15 min in alkali transfer buffer (0.25 M NaOH, 1.5 M NaCl). The DNA was

transferred, in alkali transfer buffer, to a nylon membrane (Hybond N+, Amersham). The membrane was prehybridized for 3 h at 65°C in a solution containing 4X SET (3 M NaCl, 0.6 M Tris-HCl pH 7.4, 40 mM EDTA), 0.1 % $Na_4P_2O_7$, 0.2% SDS, 0.1% heparin. Hybridization was carried out at 65°C in a solution containing 4X SET, 0.1% $Na_4P_2O_7$, 0.2 % SDS, 10% dextran sulfate and an *Arabidopsis* LTP gene fragment labeled with [α-^{32}P]dCTP by random priming. The membranes were washed for 15 min at 65°C in 2X SSC, 0.1% SDS, 15 min at 65°C in 1X SSC, 0.1% SDS, and 15 min at 65°C in 0.1X SSC, 0.1%SDS.

Production of LTP promoter - ß-glucuronidase constructs - Synthetic oligonucleotides homologous to portions of the promoter and 5' untranslated region of the genomic clone were used as primers to amplify the promoter fragment by PCR (Table 4-1). The upstream (5') primers (UH20, UH21, UH23) contained a recognition sequence for HindIII and the downstream (3') primer (UH19) contained a BamHI recognition sequence. The amplified product was digested with the appropriate restriction enzymes and subcloned into the corresponding sites of pBI101. Three constructs were prepared in this manner, one which contained about 1000 bp of the promoter fragment, one which contained 700 bp of the promoter region, and another which contained 280 bp of the promoter region.

Production of transgenic plants and screen for GUS activity The promoter-GUS fusion constructs were transformed into *Agrobacterium tumefaciens*, strain C58/pGV3850, by electroporation. Treated *Agrobacterium* were spread on LB plates containing kanamycin (50 μg/ml), resistant colonies were picked, and a plasmid miniprep was performed to ascertain the presence of the plasmid. This *Agrobacterium* was then used to transform *Arabidopsis thaliana* var. RLD, by the root transformation method described by Valvekens, *et al.* (1988). Few plants produced roots, and thus were allowed to set seed in culture. The seed were sown on MS plates containing kanamycin (50 μg/ml). Plants which were resistant to kanamycin were transplanted to pots as soon as their primary leaves developed and these plants were allowed to set seed. Leaves from transgenic plants were homogenized in GUS lysis buffer (50 mM Na PO_4, pH 7.0, 1 mM EDTA, 0.1% Triton X-100, 0.7% ß-mercaptoethanol) containing the chromogenic substrate 5-bromo-4-chloro-3-indolyl ß-D-glucuronide (X-gluc) and incubated at 37°C. The samples were checked visually for appearance of blue color.

Histochemical localization of GUS activity - Plant tissue; leaves, stems, petioles, siliques, flowers, and roots, were removed from the plant and placed in 100 mM phosphate buffer, pH 7.0, containing 1 mM spermidine and vacuum infiltration was carried out for 15 minutes. Tissue was then incubated at 37°C with a solution of 0.5 M KeFCN, 0.01% Triton-X100, 50 mM $NaPO_4$, pH 7.0, 10 mM ß-mercaptoethanol, 1 mM EDTA, 2 mM X-gluc, until a blue color appeared (usually

Table 4-1. Primer sequences used to amplify promoter fragments from ALTP1.

UH21	ACCAAAAAGCTTAATGTATTTTGGTCGAAT
UH23	ACCAAAAGCTTTTCTTATTAGAGTCATGT
UH20	ACCAAAAAGCTTAATCTCAAAACCAAAGTC
UH19	ACCAAAGGATCCATATTGATCTCTTAGGTA

after 5 hours). Following incubation with the substrate, dehydration and embedding were carried out essentially as described in De Block and Debrouwer (1992), except that the activator concentration in the embedding step was decreased from 0.8% to 0.6%. Eight micrometer sections were cut with a dry glass knife and sections were flattened on a drop of water. Sections were viewed using bright field microscopy on an Axiophot microscope (Zeiss).

Wounding and pathogen infection - Leaves of three week old plants were mechanically wounded using a Dr. Scholl's callous remover. Two hours after wounding, both wounded and non-wounded leaves were removed from plants, and leaves were stained with X-Gluc. Leaves of three week old plants were also infected with the *Arabidopsis* fungal pathogen, *Erisyphe cruciferae*. Inoculation was carried out by rubbing spores from an infected *Arabidopsis* leaf onto a test leaf. Plants which were inoculated with fungus were marked with a drop of nail polish on the tip of the leaf. Two days after infection, leaves were removed from the plant and stained with X-Gluc.

RESULTS

Cloning and characterization of an Arabidopsis LTP (ALTP1) gene - An *Arabidopsis* LTP genomic clone was isolated by screening a genomic library made from *Arabidopsis* leaf tissue (J.C. Schneider, unpublished) with a labeled

Arabidopsis LTP cDNA (chapter 5 of this dissertation; Genbank accession #M80566).

To determine the complexity of LTP genes in *Arabidopsis*, genomic DNA was digested with a variety of restriction enzymes and hybridized with the labeled *Arabidopsis* LTP cDNA. There are no cleavage sites for BamHI, HindIII, KpnI, and XbaI within the DNA sequence (as determined from sequence analysis of the genomic clone. Thus, one band would be expected on a Southern if there were one gene. There are cleavage sites for EcoRV and PvuII near the middle of the coding sequence. Two bands would be expected on a Southern if there were only one gene. Under stringent hybridization and washing conditions, two to four bands of different intensities were observed (Fig. 4-1), indicating that there are at least two LTP genes in *Arabidopsis*.

A 1950 bp fragment, which included 1150 bp of the 5' untranslated region/promoter and 500 bp coding region was sequenced (Fig. 4-2). The deduced amino acid sequence exactly matched that of the *Arabidopsis* LTP cDNA (chapter 5 of this dissertation). Sequence comparison indicated that the coding region contained a 130 bp intron, from position 396 to 526. The sequence exactly matched that of the previously identified ALTP cDNA except for one Val to Gly difference at position 528. This change is due to a one nucleotide change within the coding sequence (the conversion of T→G in the second position of the codon). The 5' untranslated region of the LTP gene showed no overall sequence homology to GenBank Release 70 listings, but several small regions were found which are

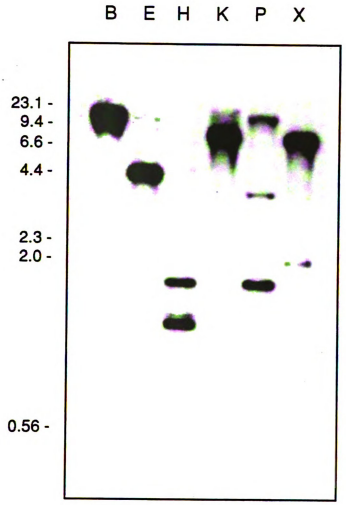
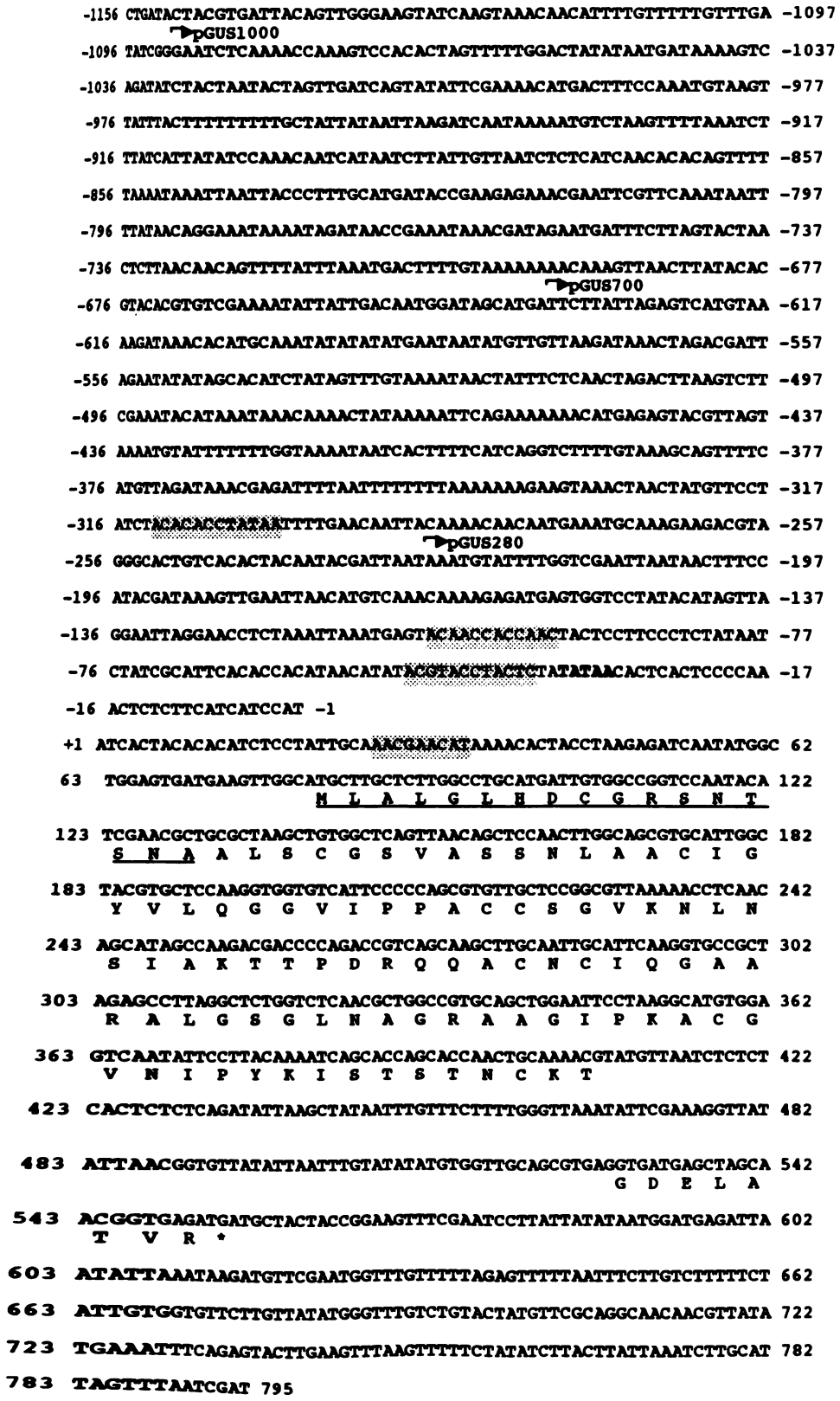


Figure 4-1. Southern blot of *Arabidopsis* genomic DNA probed with an *Arabidopsis* LTP cDNA.

Aliquots of *Arabidopsis* genomic DNA (1.7 ug) were digested with BamHI (B), EcoRV (E), HindIII (H), KpnI (K), PvuII (P), and XbaI (X), separated on an agarose gel, transferred to a nylon membrane, and hybridized with a labeled ALTP cDNA. The blot was hybridized and washed under high stringency conditions. Size markers on the left are in kilobases.

Figure 4-2. Sequence of ALTP1 promoter and coding region.

The putative transcription start site is at position +1 and a putative TATA box is given in **bold** letters. The deduced amino acid sequence is shown below the nucleotide sequence which contains a 130 bp intron from positions 396-526. The signal peptide is underlined. Sequence elements corresponding to phenylpropanoid promoters are indicated by shaded boxes. The regions used for the various ALTP1 promoter-GUS fusion are marked with an arrow (⌐▸). (This sequence was determined by U. Hecht).


-1156 CTGATACTACGTGATTACAGTTGGGAAGTATCAAGTAAACAACATTTTGTTTTTGTTTGA -1097
pGUS1000
-1096 TATCGGGAATCTCAAAACCAAAGTCCACACTAGTTTTTGGACTATATAATGATAAAAGTC -1037
-1036 AGATATCTACTAATACTAGTTGATCAGTATATTCGAAAACATGACTTTCCAAATGTAAGT -977
-976 TATTTACTTTTTTTTTGCTATTATAATTAAGATCAATAAAAATGTCTAAGTTTTAAATCT -917
-916 TTATCATTATATCCAAACAATCATAATCTTATTGTTAATCTCTCATCAACACACAGTTTT -857
-856 TAAAATAAATTAATTACCCTTTGCATGATACCGAAGAGAAACGAATTCGTTCAAATAATT -797
-796 TTATAACAGGAAATAAAATAGATAACCGAAATAAACGATAGAATGATTTCTTAGTACTAA -737
-736 CTCTTAACAACAGTTTTATTTAAATGACTTTTGTAAAAAAAACAAAGTTAACTTATACAC -677
pGUS700
-676 GTACACGTGTCGAAAATATTATTGACAATGGATAGCATGATTCTTATTAGAGTCATGTAA -617
-616 AAGATAAACACATGCAAATATATATATGAATAATATGTTGTTAAGATAAACTAGACGATT -557
-556 AGAATATATAGCACATCTATAGTTTGTAAAATAACTATTTCTCAACTAGACTTAAGTCTT -497
-496 CGAAATACATAAATAAACAAAACTATAAAAATTCAGAAAAAAACATGAGAGTACGTTAGT -437
-436 AAAATGTATTTTTTTGGTAAAATAATCACTTTTCATCAGGTCTTTTGTAAAGCAGTTTTC -377
-376 ATGTTAGATAAACGAGATTTTAATTTTTTTTAAAAAAAGAAGTAAACTAACTATGTTCCT -317
-316 ATCTACACACCTATAATTTTGAACAATTACAAAACAACAATGAAATGCAAAGAAGACGTA -257
pGUS280
-256 GGGCACTGTCACACTACAATACGATTAATAAATGTATTTTGGTCGAATTAATAACTTTCC -197
-196 ATACGATAAAGTTGAATTAACATGTCAAACAAAAGAGATGAGTGGTCCTATACATAGTTA -137
-136 GGAATTAGGAACCTCTAAATTAAATGAGTACAACCACCAACTACTCCTTCCCTCTATAAT -77
-76 CTATCGCATTCACACCACATAACATATACGTACCTACTCTATATAACACTCACTCCCCAA -17
-16 ACTCTCTTCATCATCCAT -1
+1 ATCACTACACACATCTCCTATTGCAAACGAACATAAAACACTACCTAAGAGATCAATATGGC 62
63 TGGAGTGATGAAGTTGGCATGCTTGCTCTTGGCCTGCATGATTGTGGCCGGTCCAATACA 122
M L A L G L H D C G R S N T
123 TCGAACGCTGCGCTAAGCTGTGGCTCAGTTAACAGCTCCAACTTGGCAGCGTGCATTGGC 182
S N A A L S C G S V A S S N L A A C I G
183 TACGTGCTCCAAGGTGGTGTCATTCCCCCAGCGTGTTGCTCCGGCGTTAAAAACCTCAAC 242
Y V L Q G G V I P P A C C S G V K N L N
243 AGCATAGCCAAGACGACCCCAGACCGTCAGCAAGCTTGCAATTGCATTCAAGGTGCCGCT 302
S I A K T T P D R Q Q A C N C I Q G A A
303 AGAGCCTTAGGCTCTGGTCTCAACGCTGGCCGTGCAGCTGGAATTCCTAAGGCATGTGGA 362
R A L G S G L N A G R A A G I P K A C G
363 GTCAATATTCCTTACAAAATCAGCACCAGCACCAACTGCAAAACGTATGTTAATCTCTCT 422
V N I P Y K I S T S T N C K T
423 CACTCTCTCAGATATTAAGCTATAATTTGTTTCTTTTGGGTTAAATATTCGAAAGGTTAT 482
483 ATTAACGGTGTTATATTAATTTGTATATATGTGGTTGCAGCGTGAGGTGATGAGCTAGCA 542
G D E L A
543 ACGGTGAGATGATGCTACTACCGGAAGTTTCGAATCCTTATTATATAATGGATGAGATTA 602
T V R *
603 ATATTAAATAAGATGTTCGAATGGTTTGTTTTTAGAGTTTTTAATTTCTTGTCTTTTTCT 662
663 ATTGTGGTGTTCTTGTTATATGGGTTTGTCTGTACTATGTTCGCAGGCAACAACGTTATA 722
723 TGAAATTTCAGAGTACTTGAAGTTTAAGTTTTTCTATATCTTACTTATTAAATCTTGCAT 782
783 TAGTTTAATCGAT 795

conserved among promoters of phenylpropanoid biosynthetic genes (Figs. 4-2, 4-3). A putative TATA box was found 117 bp from the translation initiation codon. The putative TATA box had 75% homology to the consensus TATA sequence TG/CTATAT/AA. A proposed transcription initiation site was determined by comparison to transcription start sites of known plant genes (Joshi, 1987). An A is at the transcription initiation site in 85% of genes examined, in the context CTCATCA. This sequence closely resembles the sequence TCCATCA, which is found in the ALTP1 gene.

Production of transgenic Arabidopsis containing the LTP promoter:ß-glucuronidase fusion - To determine the expression pattern of LTP, various size fragments of the LTP promoter were cloned into the binary vector pBI101 in front of a promoterless ß-glucuronidase (GUS) gene. The resulting constructs (Fig. 4-4) were transformed into *Arabidopsis* by the root transformation method (Valvekens, *et al.*, 1988). Control plants containing GUS driven by the CaMV promoter were also generated. Leaves from regenerated plants were homogenized and checked visually for the appearance of a blue color in the presence of X-Gluc. Four lines containing the 1 kb promoter fragment which had the highest levels of color formation were chosen for further study. These lines were designated LTP-GUS 1A, 1B, 1C, and 1D. Two strains containing the 700 bp fragment and one strain containing the 280 bp fragment also displayed color formation. These lines were named LTP-GUS 700A, LTP-GUS 700B, and LTP-GUS 280A, respectively (See

BOX 1	*Arabidopsis* LTP1	-314	ACACACCTATAA
	Arabidopsis LTP1	-52	ACGTACCTATAA
	Parsley PAL	-119	TCTCACCTACCC
	Bean PAL	-89	ACCCACCTACCA
	Bean PAL	+113	ACTCACCTACCC
	Arabidopsis PAL	-435	ACACACCTACTC
	Arabidopsis PAL	-359	TCTCACCAACCG
	Arabidopsis PAL	-64	GCTTACCTACCA
	CONSENSUS		‡CTCACCTACC‡
BOX 2	*Arabidopsis* LTP1	-110	ACAACCACCAAC
	Parsley PAL	-208	CCAACAAACCCC
	Bean PAL	-157	TCCACCAACCCC
	Arabidopsis PAL	-1631	CCAACACACCAC
	Arabidopsis PAL	-109	TCAACCAACTCC
	CONSENSUS		‡CAAC‡AACC‡C
BOX 3	*Arabidopsis* LTP1	+26	AACGAACAT
	Bean CHS	+23	AACCAACAA
	Arabidopsis PAL	+23	AACCAACAA
	CONSENSUS		AACCAACAT

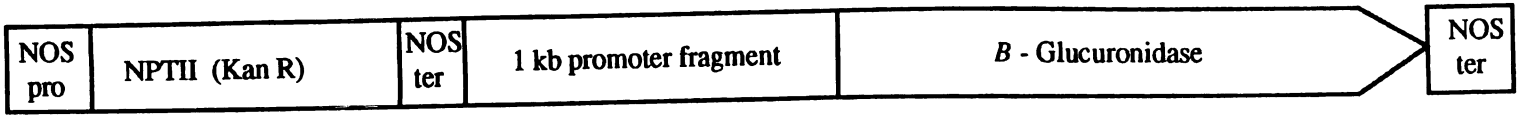
Figure 4-3. Comparison of putative regulatory sequences in ALTP1 and in genes of the general phenylpropanoid pathway.

Putative regulatory sequences from bean, parsley and *Arabidopsis* phenylalanine ammonia-lyase and bean chalcone synthase were aligned with the sequences found in the ALTP1 clone. Matches of at least 5/8 (box 1), 3/5 (box 2), or 2/3 (box 3) are shown in the consensus sequence as full size letters. The positions of the sequences are given relative to the proposed transcription start sites.

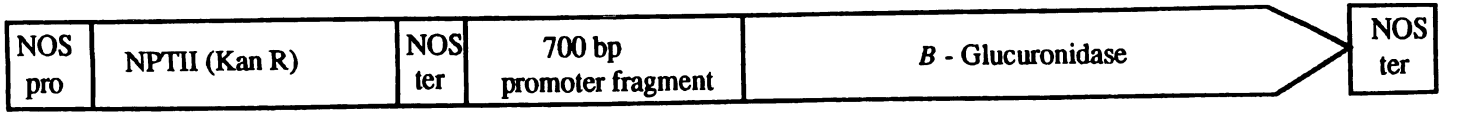
Figure 4-4. ALTP1- ß-glucuronidase constructs used to produce transgenic plants

1 Kb, 700 bp, and 280 bp fragments of the promoter and 5' untranslated region of the ALTP1 clone were placed in front of a promoterless ß-glucuronidase gene in pBI101. These constructs were transformed into *Agrobacterium tumefaciens* and subsequently used to produce transgenic plants via the root transformation method. (Constructs and transgenic plants were produced by U. Hecht).

A. pALTP1-1000



B. pALTP1-700



C. pALTP1-280

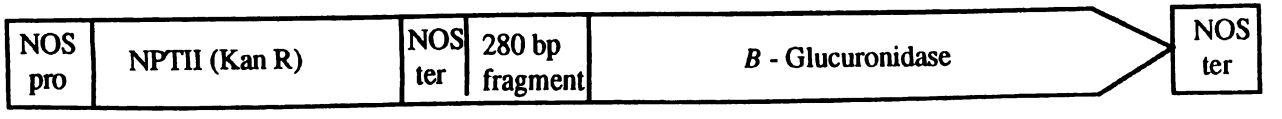


Table 4-2 for a summary).

Expression during early seedling development - Seed from transgenic plants were plated on MS medium containing kanamycin and seedlings were stained with X-Gluc. GUS staining was strong in young seedlings which had recently emerged from their seed coats (3 days after planting) in the hypocotyl region adjacent to the root (Fig. 4-5A). There was also light staining in the cotyledon. At 5 days after planting, the entire cotyledons were weakly stained, with more intense staining in the vascular tissue (Fig. 4-5B). GUS staining was also seen in the shoot meristem, in the primary leaves and in the stipules (Fig. 4-5C). As the seedling developed, GUS staining in the cotyledon decreased and eventually was confined to the tip of the cotyledon (Fig. 4-5D,F) and as the primary leaves matured, GUS staining became localized to the tips of these structures as well (Fig. 4-5E). After 10 days, staining was confined mainly to the stipules, with weak staining in the shoot meristem (Fig. 4-11A). Sections through the shoot meristem of 10 and 14 day old seedlings (Fig. 4-6) showed no staining in this region, but intense staining was observed in the stipules. No staining was seen in emerging roots, but as the roots developed, GUS staining was seen at the base of lateral roots (Fig. 4-7A,B). Staining was occasionally observed at the tip of lateral roots as they developed (Fig. 4-7C).

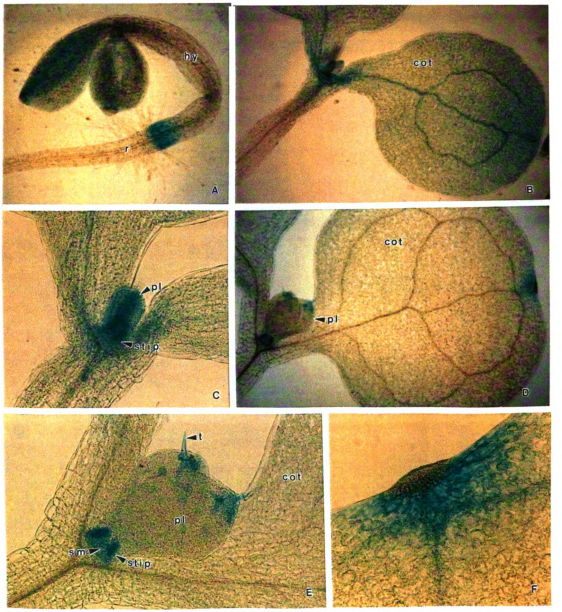
TABLE 4-2. Transgenic lines used for histochemical analysis of ALTP1 activity.

	Promoter Length (bp)	Relative Staining Intensity
LTP-GUS 1A	1000	+++
LTP-GUS 1B	1000	++
LTP-GUS 1C	1000	++
LTP-GUS 1D	1000	+
LTP-GUS 700A	700	++
LTP-GUS 700B	700	++
LTP-GUS 280A	280	++

Figure 4-5. Transgenic *Arabidopsis* seedlings after X-Gluc staining at different developmental stages.

A. ALTP1 activity in 3 day old seedling.
B. ALTP1 activity in 5 day old seedling.
C. ALTP1 activity in the shoot meristem, stipules, and primary leaves of a 5 day old seedling.
D. ALTP1 activity in 6 day old seedling. The labeling in the cotyledon is confined to the tip.
E. ALTP1 activity in the shoot meristem, stipules, and primary leaves of a 6 day old seedling.
F. ALTP1 activity near lignified tissue at tip of cotyledon.

co, cotyledon; hy, hypocotyl; pl, primary leaf; r, root; sm, shoot meristem; stip, stipule; t, trichome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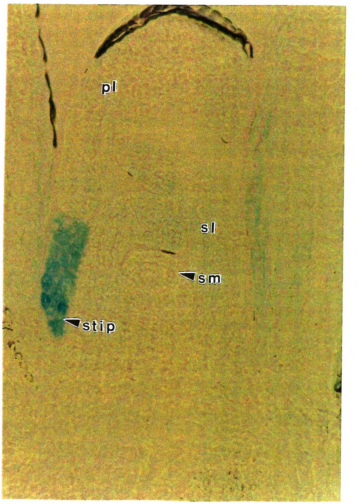
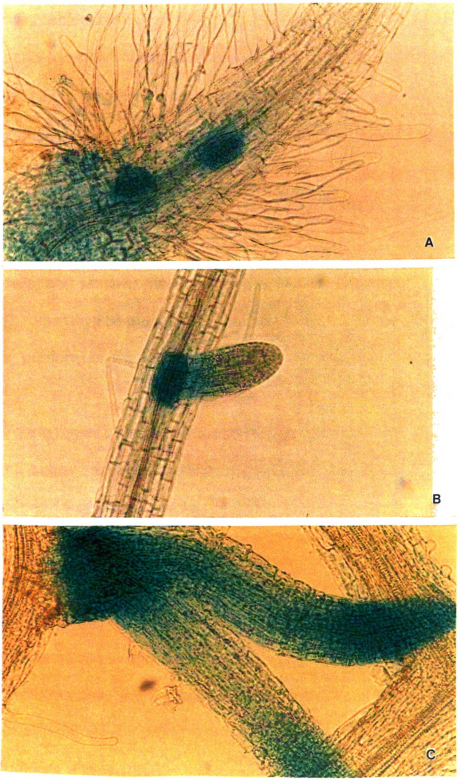


Figure 4-6. Longitudinal section through shoot meristem of 14 day old seedling. pl, primary leaf; sl, secondary leaf; sm, shoot meristem; stip, stipule

Figure 4-7. ALTP1 activity in root tissue.
A. Lateral root forming on root of 5 day old seedling.
B. Lateral root forming on 5 day old seedling.
C. Lateral root of 14 day old seedling.


A
B
C

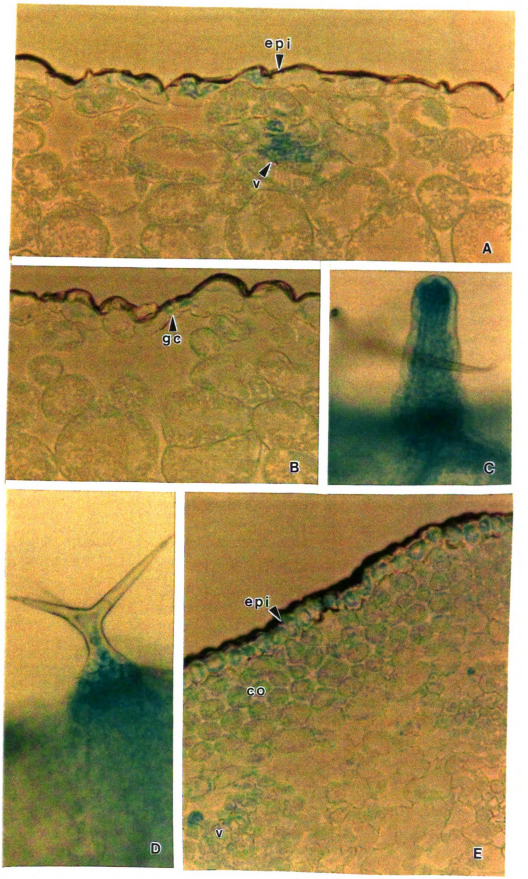
Expression of GUS in the adult plant - Tissue from kanamycin resistant plants was collected, stained with X-Glu, and either observed directly or embedded in plastic and sectioned. Analysis of leaf tissue at 2, 3, and 4 weeks after planting shows that GUS staining is most prominent in vascular tissue (Fig. 4-8A), guard cells (Fig. 4-8B), hydathodes (Fig. 4-8C), and trichomes and their associated basal cells (Fig. 4-8D). Weak staining was also observed in regular epidermal cells (Fig. Fig. 4-8A). Stem tissue was taken and analyzed from three distinct regions of the stem; at the base, from the middle, and from the top. Staining of epidermal cells, cortical cells, and vascular tissue was observed in the top portions of the stem (Fig. 4-8E). Staining was most prominent in the epidermal cells. Sections through the lower portions of the stem revealed little or no visible staining (data not shown).

Young flower buds (stage 6, Smyth *et al.*, 1990) showed no GUS staining (Fig. 4-9A), but as the flower developed, staining occurred. In the newly opened flower (stages 11-12), weak staining was observed in the stigma (Fig. 4-9B), and in the mature flower (stage 15), intense staining was observed in the stigma (Fig. 4-9F). Anthers of stage 11-12 flowers did not exhibit staining (Fig. 4-9C), but pollen grains of more mature flowers were intensely stained (fig. 4-9G). There was little or no detectable staining in floral nectaries of stage 11 flowers (Fig. 4-9D), but as the flowers matured, staining developed in these structures (Fig. 4-9E). Whole mounts of siliques shows strong staining is localized to the base and the tip of these structures (Fig. 4-10A,B). There was weak staining observed in the ovary

Figure 4-8. ALTP1 activity in leaf and stem tissue.

A. Longitudinal section through 4 week, fully expanded leaf.
B. Longitudinal section through 4 week, fully expanded leaf.
C. Hydathode from 3 week old leaf.
D. Trichome from 3 week old leaf.
E. Cross section through stem of 6 week old plant. Section taken from top of stem, near flowers.

co, cortical cells; epi, epidermis; gc, guard cell; v, vascular tissue


epi
v
A
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B
C
D
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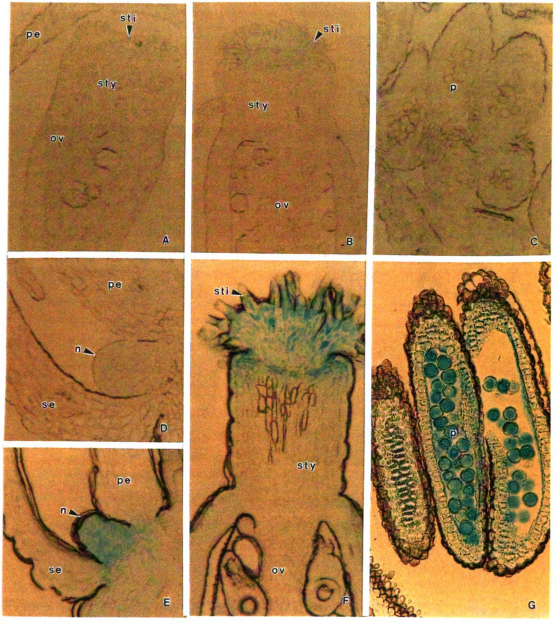
co
v
E

Figure 4-9. ALTP1 activity in flowers.

A. Longitudinal section through gynoecium of flower bud (stage 6, Smyth, *et al.*, 1990).
B. Longitudinal section through stigma and style of flower (stage 11).
C. Longitudinal section through anthers of stage 11 flower.
D. Longitudinal section through base of flower (stage 11).
E. Longitudinal section through base of mature flower (stage 15).
F. Longitudinal section through the stigma and style of mature flower (stage 15).
G. Longitudinal section through the anther of mature flower (stage 15).

n, nectary; ov, ovary; p, pollen; pe, petal; se, sepal; sti, stigma; sty, style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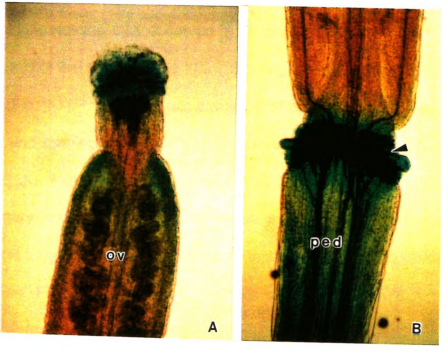


Figure 4-10. ALTP1 activity in siliques.
A. Tip of silique.
B. Base of silique. The arrowhead is pointing at zone of sepal and petal abscission.

ov, ovary; ped, pedicel

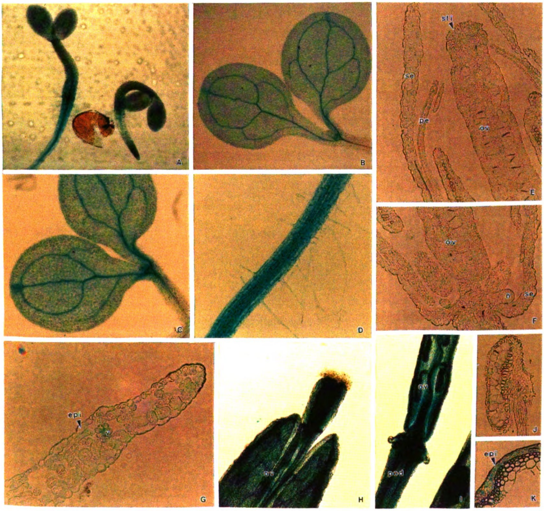
walls (Fig. 4-10A).

Control plants transformed with pBI101, which contains a CaMV 35S-GUS fusion, are shown in figure 4-11. The 35S promoter is active in most tissue and cell types. In three day seedlings, staining is observed over the cotyledons, hypocotyl and root (Fig. 4-11A). At five days (Fig. 4-11B) and six days (Fig. 4-11C), staining is observed throughout the cotyledon, and is especially prominent in the vascular tissue). Prominent staining is also observed in the root (Fig. 4-11D). Light staining was observed in all cell types of the floral organs (Fig. 4-11E,F) and of leaf tissue (Fig. 4-11G). Staining was also observed throughout the silique (Fig. 4-11H,I). No specific staining was observed in the pollen grains (Fig. 4-11J), and staining was present in the epidermal and cortical cells of stem tissue (Fig. 4-11K). Light staining was also observed in stem pith cells (data not shown). Preliminary analysis of GUS expression in plants containing the shortened promoter fragments (Table 4-1), indicate that the activity of the promoter is similar in all lines. Figure 4-12 represents analysis of 10 day old seedlings with truncated promoter fragments.

Wounding and pathogen infection - Leaves of transgenic and control plants were mechanically wounded and stained for GUS activity 2 hours after wounding. There was no noticeable increase in staining after this treatment. Transgenic and control plants were treated with the fungus, *Erisyphe cruciferae*, and leaves were collected 48 h after infection. There was a marked increase in staining in the cells

Figure 4-11. Activity of CaMV 35S promoter in transgenic *Arabidopsis*.

A. 3 day old seedlings.
B. 5 day old seedling.
C. 6 day old seedling.
D. Primary root from 6 day old seedling.
E. Longitudinal section through floral organs. The staining is light, but is present in all cells types at an equal intensity.
F. Longitudinal section through base of flower. Staining is light, but present in all cell types at an equal intensity.
G. Longitudinal section through a leaf. Staining is present in all cell types.
H. Tip of silique.
I. Base of silique.
J. Longitudinal section through anther.
K. Cross section of a stem.



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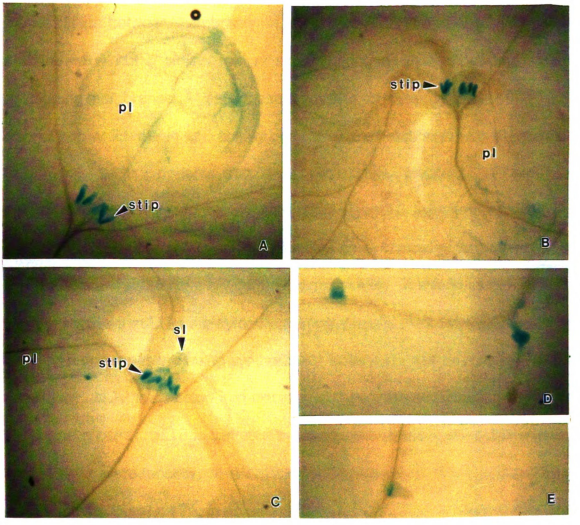
I
J
epi
K

Figure 4-12. ALTP1 activity in lines containing 1 Kb, 700 bp, and 280 bp promoter fragments. Seedlings were stained at 10 days.

A. Seedling containing 1 Kb fragment.
B. Seedling containing 700 bp fragment.
C. Seedling containing 280 bp fragment.
D. Roots of seedling containing 280 bp fragment.
E. Roots of seedling containing 700 bp fragment.

pl, primary leaf; sl, secondary leaf; stip, stipule


pl
stip
A
stip
pl
B
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surrounding areas of damage and in the vascular tissue near wounded cells. These results are still very preliminary, and thus, the data is not shown here.

DISCUSSION

Lipid transfer proteins were originally isolated based on their ability to transfer lipids between membranes *in vitro*. It has long been assumed that the proteins carry out a similar role *in vivo*, carrying newly synthesized lipids to developing membranes. A yeast phosphatidylinositol transfer protein (PI-TP) is the only LTP to which the *in vitro* activity correlates to a function *in vivo* (Bankaitis, *et al.*, 1990). It appears that this protein is involved in regulating phosphatidylinositol/phosphatidylcholine ratios of yeast Golgi membranes (Cleves, *et al.*, 1991). However, plant nsLTPs lack sequence homology to the yeast PI-TP and, thus, are likely to have a different biological role. A role in intracellular lipid transfer implies that LTPs are cytosolic and are present in all cell and tissue types in which membranes are being synthesized. However, it has been demonstrated that barley nsLTP is secreted into aleurone culture medium (Mundy and Rogers, 1986) and a carrot nsLTP is secreted by carrot embryogenic cell cultures (Sterk, *et al.*, 1991). More recently, an *Arabidopsis* LTP was shown to be localized to cell walls and was found mainly in epidermal cells in the tissues examined (chapter 3 of this dissertation; Thoma, *et al.*, 1993). A maize LTP was shown to be expressed mainly in epidermal cells and vascular strands of maize coleoptiles (Sossountzov, *et al.*, 1991), and a tobacco LTP was highly expressed in leaf epidermal cells and

in the shoot apical meristem (Fleming, *et al.*, 1992). In *Arabidopsis* (chapter 3 of this dissertation; Thoma, *et al.*, 1993), spinach (chapter 2 of this dissertation; Bernhard, *et al.*, 1991), maize (Sossountzov, *et al.*, 1991), and tobacco (Fleming, *et al.*, 1992), LTP is found to be localized or expressed in the aerial portions of the plant, with little or no localization or expression in root tissue. Clearly, the cell-type and tissue specific localization of plant nsLTPs is not consistent with a direct role of these proteins in intracellular lipid transfer.

A genomic clone corresponding to an *Arabidopsis* nsLTP has been isolated. Analysis of the complexity of the *Arabidopsis* genome reveals the presence of at least two genes encoding for LTP, and this gene has been designated ALTP1. The presence of multiple genes is not entirely surprising, as LTP isoforms have been found in castor bean (Tsuboi, *et al.*, 1991), barley (Mundy and Rogers, 1986; R. Kalla, personal communication), tobacco (Koltunow, *et al.*, 1990; Fleming, *et al.*, 1992), and wheat (Dieryck, *et al.*, 1992), and Southern analysis indicates that there may be several LTP genes in maize (Tchang, *et al.*, 1989) and tomato (Torres-Schumann, *et al.*, 1992). The deduced amino acid sequence of the ALTP1 clone showed an exact match to that of a previously isolated cDNA clone (chapter 5 of thisdissertation), except for one Val to Gly conversion at position 528. This could indicate that we have cloned a gene other than the one corresponding to the previously isolated cDNA. It is also possible that the difference observed is due to an error in reading the DNA sequence, as the change is due to the conversion of one base pair (T→G) in the middle of the codon.

To better understand plant nsLTPs and to provide insight into their possible function, transgenic plants containing ALTP1 promoter-ß-glucuronidase fusions were produced. The regulation of a reporter gene by a heterologous promoter in transgenic plants has been shown to be an accurate representation of the intrinsic regulatory properties of the introduced promoter (Benfey and Chua, 1989; Bevan, *et al.*, 1989). As anticipated in these experiments, there was variation in the relative levels of expression in different transformed lines. Line LTP-GUS 1A had high relative levels of GUS expression, LTP-GUS 1D had a low level of expression, and LTP-GUS 1B and C had moderate expression levels. Transgenic lines containing shorter promoter fragments also exhibited moderate expression levels. These differences were probably due to positional effect from insertion of the gene construct in various sites of the target genome. The problem was overcome by looking at the expression pattern of several independently transformed lines. The overall pattern of expression did not differ between lines containing the same transgene.

Analysis of the temporal and spatial activity of the ALTP1 promoter demonstrated that it was active in very specific cell and tissue types. Certain aspects of the pattern observed were similar to the pattern of cells and tissues in which phenylpropanoid biosynthesis occurs and in which the products of the phenylpropanoid biosynthetic pathway reside. Phenylpropanoids are a class of plant natural products derived from phenylalanine. These compounds play important roles in plant development and in protection against environmental

stress. For instance, flavonoids are pigments and UV protectants in epidermal cells, lignin is the major structural component in xylem cell walls, and suberin is a lipophilic substance commonly found in the casparian strip of the endodermis (Esau, 1977). The induction of lignin in wheat (Moerschbacher, *et al.*, 1990), suberin deposition in potato (Roberts and Kolattukudy, 1989) and the accumulation of phenylpropanoid derived phytoalexins help protect a plant against mechanical damage and microbial attack (Dixon and Lamb, 1990).

The ALTP1 promoter was active in early seedling development. The pattern of staining observed in the cotyledon and primary leaves follows the pattern of xylem differentiation. Xylem development commonly occurs at the basal portion of the leaf, then progresses in an acropetal manner. In leaves of adult plants, there was also staining associated with vascular tissue. More detailed analysis of mature leaves needs to be carried out to determine if vascular expression proceeds in a manner consistent with xylem differentiation.

In the developing seedling, staining was also prominent in emerging lateral roots. Lateral roots arise from the pericycle and their emergence causes damage to cortical cells. This results in an opening into the interior of the parent root, and this "wound" may induce lignin and/or suberin deposition. Staining was also observed in the stipules of young seedlings at least until two weeks of age. It is difficult to assign a role for LTP in stipules, as this organ has no known function. Staining was observed in the shoot apical meristem of developing seedlings. A tobacco LTP has been shown to be highly expressed in the shoot meristem, and

in situ hybridization experiments show that the expression is located mainly in the L1 layer. To see if the ALTP1 was active in a specific cell type, sections were made through the shoot meristem of stained 10 d and 14 d seedlings. Although there was weak staining observed in the shoot meristem in whole mounts of 10 d seedling, expression of the ALTP1 promoter was not detected in sectioned tissue. It is possible that the activity is at a low level which cannot be detected in thin sections. Sections through younger tissue, where the meristematic acivity is more prominent, need to be analyzed.

In the mature plant, the ALTP1 promoter was active in cells containing a thick cuticle layer; leaf and stem epidermal cells, guard cells, and hydathodes. Cutin is a polymer found associated with the cell wall of cells on the outer portion of the aerial parts of plants, and phenolic acids such as *p*-coumarate and ferulic acid are structural components (Riley and Kolattukudy, 1975). Staining observed in stems was weak, but was more prominent near the top of the stem. This type of developmental expression in which LTP is expressed at higher levels in the younger part of the plant was also observed in tobacco (Fleming *et al.*, 1992). This differential expression was quite apparent in bulk stained stem tissue, but more difficult to discern in sectioned tissue, and further studies to quantitate the GUS activity by a fluorometric assay (Jefferson, *et al.*, 1987) need to be carried out.

The ALTP1 promoter was active during floral development. At the stage of development where the sepals enclose the flower bud (stage 6, Smyth, *et al.*,

1990), no staining was observed. At stage 11-12, when the stigmatic papillae have appeared and the petals are level with long stamens, staining is observed on the stigma surface. By stage 15, when the stigma extends above the long anthers, staining is observed in the stigma surface, the floral nectaries, and in developing pollen grains. Floral nectaries are covered with a cuticular substance and stigma cells can produce a secretion containing lipids and phenolic compounds such as anthocyanins, flavonoids, and hydroxycinnamic acids (Martin and Brewbaker, 1971). Pollen grains are covered with a polymerized lipid material, sporopollenin. The chemical nature of sporopollenin is largely unknown, but there is evidence that it may contain phenolic materials (Kolattukudy, 1980). Pigment synthesis also occurs in developing pollen grains. Staining was observed at the base of the ripening silique in the abscission zone of sepals and petals. This pattern is consistent with that seen of an *Arabidopsis* phenylalanine ammonia-lyase (PAL) promoter (Ohl, *et al.*, 1990). Weak staining was also observed in the silique walls.

Analysis of the promoter and 5' untranslated region of the ALTP1 gene revealed the presence of several sequence elements commonly found in promoters of biosynthetic genes of the phenylpropanoid pathway (Cramer, *et al.*, 1989; Lois, *et al.*, 1989; Ohl, *et al.*, 1990). Two elements (boxes 1 and 2) are homologous to sequences in bean, parsley, and *Arabidopsis* PAL promoters. In parsley, these sequence elements have been shown to display elicitor-inducible and light-inducible footprints *in vivo*. A 9 bp AC rich element which is found in the 5' untranslated regions of an *Arabidopsis* PAL gene and a bean chalcone synthase

(CHS) gene was also observed in this region of the ALTP1 gene. These sequence data, along with the GUS expression pattern are consistent with a role for the *Arabidopsis* nsLTP in phenylpropanoid metabolism or regulation of expression by the same or similar mechanisms.

The role of possible regulatory sequences can be tested by examining the staining pattern of plants containing truncated versions of the ALTP1 promoter. Two of these shortened promoters, one containing a 700 bp fragment and the other a 280 bp fragment of the promoter, have been transformed into plants. There has not been extensive analysis of these plants, although preliminary data suggests that expression patterns are similar to that observed with the 1 kb promoter in 4 d and 10 d seedlings. To test the putative regulatory sequences described in this paper, however, shorter promoter fragments and site specific mutagenesis in specific regulatory sites must be carried out. Lignification and suberization are induced in a plant upon wounding or pathogen infection. Preliminary data suggest that ALTP1 may indeed be activated upon wounding. Analysis of transgenic plants containing the ALTP1-GUS fusion show that there is no GUS induction 2 hours after mechanical damage to the plant. However, 48 h after infection with the fungal pathogen *Erysiphe cruciferae*, there is a significant increase in staining around areas of cell damage in the infected leaves and in the vascular tissue near the area of cell damage. The method of inoculation involves rubbing an infected leaf over the test leaf, so it is not possible to tell if the cell damage is due to fungal invasion, or to mechanical damage due to the inoculation

technique. It will be important to repeat these tests and to more fully assess the effect of wounding and pathogen infection on the induction of ALTP1, using both qualitative (histochemical) and quantitative (fluorometric) techniques.

A possible limitation to the GUS expression studies described here arises from the possible role of nsLTPs in plant stress responses. If ALTP1 is induced by stress, the act of removing the tissue from the plant and adding it to the staining solution may be stressful enough to trigger induction of the gene. Thus I may be looking the stress induced pattern of expression. It may be important to pre-fix the tissue in a fixative such as paraformaldehyde, to look at normal, non-stress induced pattern of expression.

Activation of ALTP1 by biotic and abiotic stress requires further study. The expression of a tomato nsLTP was shown to be induced by salt stress (Torres-Schumann, *et al.*, 1992), and proteins with regions of homology to LTPs have been shown to be induced by drought stress. Analysis of transgenic *Arabidopsis* plants which contain a reduced level of LTP due to the presence of an *Arabidopsis* LTP cDNA in reverse orientation behind the CaMV 35S promoter, show no alteration in their wax or cutin contents under normal growth conditions (chapter 5 of this dissertation). Cutin composition from plants grown under drought conditions, where the synthesis of cuticular components is a limiting factor in plant survival, needs to be analyzed. Also it will be useful to examine the lignin composition of wounded and nonwounded antisense plants.

In conclusion, the ALTP1 promoter specifies a complex and specific

expression pattern in transgenic plants. The patterns observed, along with the presence of promoter sequences homologous to putative regulatory elements of phenylpropanoid biosynthetic genes, suggests that plant nsLTPs may be involved in phenylpropanoid metabolism or the expression of LTP genes is regulated by the same or similar mechanisms as phenylpropanoid genes.

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CHAPTER 5

ANALYSIS OF TRANSGENIC *ARABIDOPSIS* CONTAINING A REDUCED LEVEL OF A NON-SPECIFIC LIPID TRANSFER PROTEIN

ABSTRACT

A class of proteins, called lipid transfer proteins (LTPs), has been shown to mediate transfer of phospholipids between membranes *in vitro*; however, there is no evidence that these proteins catalyze lipid transfer *in vivo*. I have recently demonstrated by immunocytochemical labeling at the ultrastructural level that an *Arabidopsis* LTP has an extracellular location, with its highest concentration in the epidermal cells of aerial plant organs. The extracellular location and the cell-type and tissue-type specific location is inconsistent with the proposed role of the protein in intracellular lipid transfer. To determine the role of the protein, several transgenic plants in which the level of LTP has been reduced by the expression of an LTP antisense construct were generated. Western analysis indicated that these plants contain less than 10% of the LTP of wild type plants. The transgenic plants exhibited no visible phenotype to indicate a possible function of LTP within the plant. The location of this protein and its ability to bind hydrophobic molecules, leads to a speculation that the protein may be involved in cuticle formation. The wax and cutin composition of the antisense plants has been analyzed and there is no difference in comparison to wild type plants under normal growth conditions.

INTRODUCTION

In eukaryotic cells, lipid synthesis occurs mainly on the lumenal face of the endoplasmic reticulum (ER), and in all plant species, some lipid synthesis also occurs in the chloroplast. Although lipid biosynthesis is restricted to a few organelles within a cell, lipids are the major component of all cellular membrane systems of a cell, with different organelles containing unique and specific lipid compositions. Thus, several membranes and organelles need to import their lipid constituents. The mechanism(s) by which lipids move from their site of synthesis to other cellular organelles is unknown.

In an attempt to elucidate the mechanism by which lipid movement occurs, scientists began to search for proteins which were capable of transferring phospholipids between membranes *in vitro*. Such proteins, called lipid transfer proteins (LTPs), have subsequently been isolated from animal and plant tissue, yeast, and bacteria. LTPs which transfer specific phospholipids, such as a phosphatidylcholine transfer protein (PC-TP) and a phosphatidylinositol transfer protein (PI-TP), have been isolated from animal tissue and yeast (Wirtz, 1982; Helmkamp, 1990). Non-specific LTPs (nsLTPs), which are capable of transferring several classes of phospholipids, have been isolated from bacteria, plant and animal tissue (Wirtz, 1982; Arondel and Kader, 1990). The plant nsLTPs have been isolated from several species, including spinach (Kader, *et al.*, 1984; Bouillon, *et al.*, 1987), maize (Douady, *et al.*, 1982), barley (Mundy and Rogers, 1986), sunflower (Arondel, *et al.*, 1990), wheat (Dieryck, *et al.*, 1992) and castor

(Takishima, *et al.*, 1986). Although the plant LTPs share no obvious sequence similarity to the nsLTPs from other organisms, they have considerable similarity among themselves and they share several common features; they are soluble, basic proteins with a molecular mass around 9 kDa.

The ability of LTPs to transfer phospholipids *in vitro* has led to the proposal that LTPs act to shuttle lipids between organelles *in vivo*. However, there has been no direct evidence which supports this proposal. The only LTP to which the *in vitro* activity correlates to a function *in vivo* is a yeast PI-TP which appears to be involved in regulating the phosphatidylinositol/phosphatidylcholine ratios in yeast Golgi membranes (Bankaitis, *et al.*, 1990). It has been proposed that this protein acts not by physically transferring lipids, but by sampling the PI/PC ratio and subsequently regulating PC synthesis (Cleves, *et al.*, 1991). However, the plant nsLTPs lack sequence homology with the yeast PI-TP, and are thus likely to have a different biological role.

Based on the proposed function of intracellular lipid transfer, it has been assumed that LTPs were cytoplasmic (Arondel and Kader, 1990). However, there is much evidence that contradicts a cytosolic location. It has been reported that the maize LTP has an amino terminal extension and is synthesized on membrane bound polysomes (Tchang, *et al.*, 1988, Vergnolle, *et al.*, 1988) and that spinach LTP contains a signal peptide and is cotranslationally inserted into microsomal membranes (Bernhard, *et al.*, 1991; chapter 2 of this thesis). As these proteins lack the carboxy terminal ER retention signal, KDEL (Munro and Pelham, 1987),

they would be expected to be secreted or targeted to a specific organelle. These expectations were reinforced by reports that a secreted barley protein (Mundy and Rogers, 1986) was homologous to nsLTPs (Bernhard and Somerville, 1989) and was capable of *in vitro* lipid transfer (Breu, *et al.*, 1989), and that a carrot nsLTP was secreted by carrot embryogenic cell cultures (Sterk *et al.*, 1991). Immunocytochemical studies have shown that a maize LTP is localized to the periphery of epidermal cells, and it has been suggested that the staining is associated with the plasma membrane (Sossountzov, *et al.*, 1991). An *Arabidopsis* nsLTP has been localized to the cell wall and cutin layer in the aerial portions of the plant, and is observed mainly in epidermal cells in leaves, petioles, stems, and in the cells of the stigma surface (Thoma, *et al.*, 1993; chapter 3 of this dissertation).

The extracellular location of plant nsLTPs indicates that these proteins are not directly involved in intracellular lipid transfer. This opens the possible *in vivo* role of these proteins to speculation. The preferential accumulation of the *Arabidopsis* LTP to the epidermal cell walls is consistent with the prediction that the protein may be involved in cuticle formation (Sterk, *et al.*, 1991; Thoma, *et al.*, 1993; chapter 3 of this dissertation). The cuticle is a continuous layer of predominantly lipophilic material found on the outermost surface of the aerial parts of plants (Holloway, 1982). The cuticle is composed of cutin, an insoluble polymer composed mainly of C_{16} and C_{18} hydroxylated and epoxygenated fatty acids, and wax, which contains several classes of long chain hydrocarbons, including long

chain fatty acids (Kolattukudy, 1982). Wax and cutin monomers are synthesized in epidermal cells and are delivered to the outside of the cell where it is thought that esterification takes place (Croteau and Kolatukuddy, 1974). It is unknown how the hydrophobic monomers pass through the aqueous environment of the cell wall. Since LTPs have been shown to bind fatty acids (Rickers, *et al.*, 1985), it has been proposed that LTPs may play a role in the deposition of these aliphatic chains to the outer cell surface.

Another proposed role for nsLTPs is involved with general plant defense. A radish protein, originally isolated based on its *in vitro* antifungal activity, shares sequence homology with plant nsLTPs (Terras, *et al.*, 1992). Also, LTPs isolated from barley and maize show strong antifungal activity in *vitro* assays (Garcia-Olmeda, personal communication).

More recently, I have shown, using transgenic plants that contain an *Arabidopsis* LTP promoter:ß-glucuronidase (GUS) fusion, that the promoter is active in cells and tissues where phenylpropanoid metabolism is operative (chapter 4 of this dissertation). The GUS expression pattern, along with the presence of sequence elements homologous to putative regulatory elements of phenylpropanoid biosynthetic genes, has led to the proposal that LTP may play a role in phenylpropanoid metabolism, or that expression of LTP genes is regulated by similar mechanisms (chapter 4 of this dissertation).

To determine the role of nsLTPs *in vivo*, transgenic plants which contain an *Arabidopsis* nsLTP cDNA in reverse orientation behind the cauliflower mosaic virus

35S promoter were generated. Western analysis shows that these plants contain a greatly reduced amount of protein when compared to control plants. Apart from a delay in time of flowering, the plants containing the antisense construct, the plants had no obvious phenotypic differences in comparison to control plants. To test for the role of the protein in cuticle formation we analyzed the wax and cutin composition of control and antisense plants using gas chromatography and electron microscopy. To examine a possible role for LTP as an antifungal compound, I also tested the ability of the antisense plants to withstand pathogen attack. Experiments are also proposed to test the role of the protein in phenylpropanoid metabolism. The results of these analyses are presented here.

EXPERIMENTAL PROCEDURES

Plant growth conditions - Unless otherwise indicated, all plants were grown at 22°C under continuous fluorescent illumination (100-150 μmol m^{-2} s^{-1}) on a potting mixture of fine sphagnum:perlite:vermiculite (1:1:1).

Isolation of an Arabidopsis cDNA clone - A λGT10 *Arabidopsis* leaf cDNA library was plated on *Escherichia coli* LE392 cells and nitrocellulose plaque lifts were screened with a spinach LTP cDNA which was labeled with [α-^{32}P] dCTP by random priming (Feinberg and Vogelstein, 1983). Filters were prehybridized for 4 h at 42°C in a solution of 5X Denhardt's, 5X SSC, 0.1% SDS, and 0.5 μg/μl sonicated herring DNA. Hybridization was carried out for 16 h at 42°C in the same

solution containing the radiolabeled probe. The filters were washed twice at room temperature, first in 4X SSC, followed by 2X SSC, and once at 42°C in 2X SSC. All washes were carried out for 15 min. The resulting cDNA was subcloned into pBluescript and sequenced. All nucleic acid manipulations were carried out as described in Maniatis, *et al.* (1982).

Production of antisense constructs - To produce plants in which the amount of LTP has been reduced, three chimeric gene constructs were prepared. The constructs were made by inserting fragments of an *Arabidopsis* LTP cDNA into the binary vector, pBI121, in reverse orientation between the CaMV 35S promoter and the nos terminator. The ß-glucuronidase gene was cut out of pBI121 with the restriction enzymes SmaI and SacI. To prepare the constructs pANA1 and pANA2, the *Arabidopsis* LTP cDNA was digested with EcoRI, which made a single cut near the center of the gene, and these two fragments were subcloned into the EcoRI site of pBluescript. The LTP cDNA fragments were removed from pBluescript by restriction digests with SacI and SmaI and the resulting fragments were ligated into the corresponding sites of pBI121. To construct pANA3, which contained the full length cDNA, the cDNA was amplified from the phage by PCR using primers containing BamHI restriction sites. The PCR product was digested with BamHI and subcloned into the corresponding site of pBluescript. The resulting plasmid was then cut with SacI and EcoRV and subsequently subcloned into the SacI and SmaI sites of pBI121.

Production of transgenic plants - Each antisense construct and the vector, pBI121, were transformed into *Agrobacterium tumefaciens* C58/pGV3850 by electroporation. These *Agrobacterium* were used to transform *Arabidopsis thaliana* (vars. RLD, NO-O, ST0, and C24) by the root transformation method as described by Valvekens, et al., 1988. Seed were collected from initial transformants, sterilized and plated on Murashige-Skoog (MS) medium (Murashige and Skoog, 1962) containing kanamycin (50 μg/ml). T2 plants which were resistant to kanamycin were transplanted to pots and allowed to set seed. Segregation of kanamycin resistance in the transgenic plants was followed in this manner until all lines were homozygous for the transgene. Homozygous plants were used for all subsequent analysis.

Screening transgenic plants - Protein extracts were made from leaves of individual transformed plants by homogenizing tissue in 20 mM glycine, pH 8.4, 5 mM $MgCl_2$, 25 mM EDTA. Extracts were centrifuged twice at 10,000 X g for 10 min. Protein concentrations were determined by the BCA protein assay (Pierce Biochem, Rockford, Ill) and equal amounts of protein from transgenic and wild type plants were loaded onto 10-20% gradient SDS-polyacrylamide gels. The resolved proteins were transferred to nitrocellulose and subjected to Western analysis essentially as described by Towbin, et al. (1979) using anti- *Arabidopsis* LTP antibodies (Thoma, et al., 1993; chapter 3 of this thesis). Goat anti-rabbit IgG-alkaline phosphatase conjugate was used as the second antibody. Alkaline

phosphatase activity was shown using nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate as substrates. Over 50 independently transformed lines were screened in this manner. Plants which had low levels of protein in comparison to wild type plants and control plants which were transformed with pBI121 containing no antisense cDNA were rescreened by Western analysis. Four lines, LTP4, LTP5, LTP12, and LTP18 had the greatest reduction in LTP, and were used for subsequent analyses. T17, a transgenic line containing pBI121 without an antisense cDNA, had similar levels of LTP as wild type and was used as a control in subsequent analyses.

Growth curves - Seed from transgenic and wild type plants were sown on the potting mixture described above. For growth measurements, the fresh weight of the entire aerial portion of plants (n=10) was determined at 2 day intervals, from day 4 to day 26. To measure time of flowering, the percentage of individual plants that were flowering ($n \geq 10$) was measured each day from the time control plants started bolting (day 14) until all plants were bolting. Percent germination was determined by planting a known number of seed per pot and counting the number of resulting plants.

Gas chromatographic analysis of wax and cutin composition - Epicuticular wax was isolated by washing stems and leaves of wild type and transgenic plants twice with chloroform. The chloroform extracts were concentrated by drying under

N_2, and separated by gas chromatography on a 15 m X 0.53 mm ID Supelco SPB-1 column, using flame ionization detection.

To isolate cutin, the residual plant material from the above preparation was heated (80°C) in several changes of chloroform:methanol (1:1) for 24 hours. The tissue was then heated under reflux (75°C) in 3N methanolic HCl for 48 hours and then extracted with NaCl (0.1M):hexane (1:4). The hexane phase, which contained the methyl esters of cutin monomers, was collected, concentrated, and was separated by gas chromatography as described for wax esters.

Scanning electron microscopy - Stems from 5 week old transgenic and wild type plants were removed and segments from similar portions of the stems were viewed on an EMscope cryo stage of a JEOL 35CF scanning electron microscope.

Preparation of samples for transmission electron microscopy - Stems from 5 week old transgenic and wild type plants were removed and similar portions of the stems were fixed for 1.5 h, at room temperature in 2.5% (v/v) glutaraldehyde in 0.1 M sodium phosphate, pH 7.0. The samples were washed 3 times with phosphate buffer and post fixed for 1 h in 1% (v/v) osmium tetroxide in 0.1 M sodium phosphate, pH 7.0 at room temperature. The tissue was washed 3 times with phosphate buffer, dehydrated in a graded series of ethanol (25-100%) and infiltrated with Spurr's resin (Polysciences, Warrington, PA). Infiltration was carried out over a 48 hour period with several resin changes. Dehydration and infiltration

were carried out at room temperature. The resin was polymerized for 24 h at 65°C. Ultrathin sections (90-100 nm) were cut with a diamond knife and mounted on copper grids. On average, 40 sections of each of 5 sample preparations from each antisense line were examined. Sections were stained with a saturated uranyl acetate solution for 30 min and Reynolds lead citrate for 5 min. Sections were viewed on a Philips 201 electron microscope.

Infection of plants with fungal pathogens - Transgenic plants containing an antisense construct and plants containing the empty transformation vector were grown in pots to 2.5 weeks of age and inoculated with *Erysiphe cruciferae* by rubbing an infected leaf on the leaves of the test plant. Plants were placed in a humidified chamber for 1 hour and left at 22°C for 7 d.

RESULTS

Isolation of an Arabidopsis nsLTP cDNA clone - A cDNA corresponding to a spinach LTP was used as a probe to isolate an LTP cDNA clone from *Arabidopsis*. The sequence of the cDNA was determined (Fig 5-1) and the deduced amino acid sequence shows 49% identity to the consensus sequence (chapter 1 of this thesis) of other known LTPs (Fig. 5-2). The presence of a 17 amino acid amino terminal extension is consistent with the reports that LTPs from spinach, maize, and barley contain signal peptides.

```
  1 AAAACTACGTCACTGGAATTCGGTGAAGTTGGCATGCTTGCTCTTGGCCTGCATGATTGT
                                      M  L  A  L  G  L  H  D  C

 61 GGCCGGTCCAATACATCGAACGCTGCGCTAAGCTGTGGCTCAGTTAACAGCAACTTGGCA
     G  R  S  N  T  S  N  A  A  L  S  C  G  S  V  N  S  N  L  A

121 GCGTGCATTGGCTACGTGCTCCAAGGTTGTGTCATTCCCCCAGCGTGTTGCTCCGGCGTT
     A  C  I  G  Y  V  L  Q  G  G  V  I  P  P  A  C  C  S  G  V

181 AAAAACCTCAACAGCATAGCCAAGACGACCCCAGACCGTCAGCAAGCTTGCAATTGCATT
     K  N  L  N  S  I  A  K  T  T  P  D  R  Q  Q  A  C  N  C  I

241 CAAGGTGCCGCTAGAGCCTTAGGCTCTGGTCTCAACGCTGGCCGTGCAGCTGGAATTCCT
     Q  G  A  A  R  A  L  G  S  G  L  N  A  G  R  A  A  G  I  P

301 AAGGCATGTGGAGTCAATATTTCTTACAAAATCAGCACCAGCACCAACTGCAAAACCGTG
     K  A  C  G  V  N  I  S  Y  K  I  S  T  S  T  N  C  K  T  V

361 AGTGATGAGCTAGCAACGGTGAGATGATGCTACTACCGGAAGTTTCGAATCCTTATTATA
     S  D  E  L  A  T  V  R  *

421 TAATGGATGAGATTAATATTAAATAAGATGTTCGAATGTTTGTTTTTAGAGTTTTTAATT
481 TCTTGTCTTTTTTCTATTGTGGTGTTCTTGTTATATGGGTTTGTCTGTACTATGTTCGCAG
541 GCAACAACGTTATATGAAATTTCAGAGTACTTGAAGTTTAAGTTAAAAAAAAAAAACCGA
601 ATTC
```

Figure 5-1. Sequence analysis of the *Arabidopsis* cDNA clone.

Nucleotide sequence of *Arabidopsis* cDNA and its deduced amino acid sequence. The cDNA sequence was determined by W. Bernhard and J. Botella.

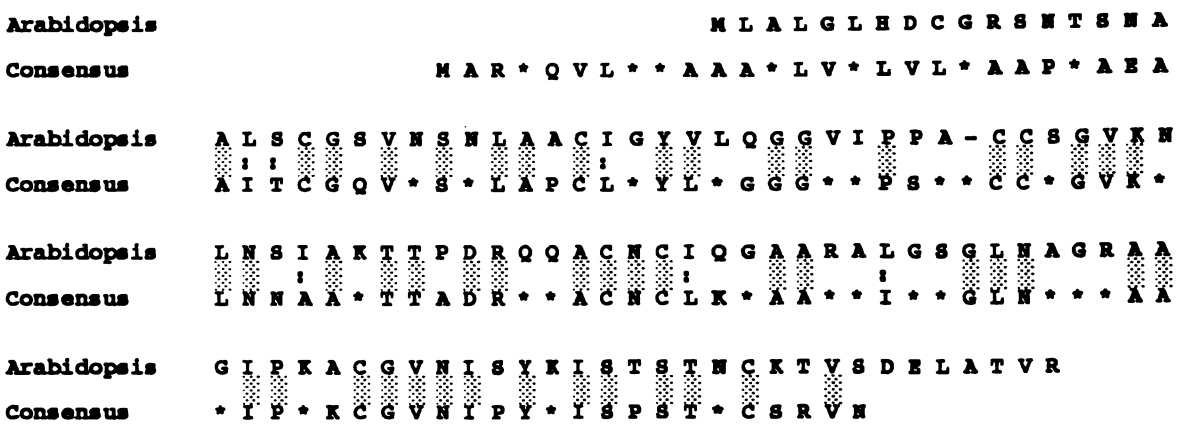


Figure 5-2. Comparison of the *Arabidopsis* LTP sequence to LTP consensus sequence.

Deduced amino acid sequence of an *Arabidopsis* LTP determined from cDNA sequence and comparison to the consensus sequence of other known LTPs (see chapter 1 of this thesis). Amino acids which are identical to the consensus sequence are connected with (⣿) and conserved substitutions are connected with (:).

Production of antisense plants - To produce plants in which the amount of LTP has been reduced, three chimeric gene constructs were prepared. As the mechanism of antisense suppression of endogenous genes is still not well understood, and there are varying reports as to the effectiveness of 3' end vs. 5' end vs. full length clone, we made constructs containing various portions of the cDNA as well as the full length clone. The constructs were made by inserting the full length *Arabidopsis* cDNA or portions of the cDNA into the binary plant transformation vector, pBI121, in reverse orientation behind the CaMV 35S promoter and the nos terminator (Fig. 5-3).

Antisense constructs and pBI121 were each transformed into *Agrobacterium tumefaciens* strain C58 by electroporation. These *Agrobacterium* were used to transform *Arabidopsis* plants by the root transformation method described by Valvekens, et al. (1988). In an attempt to increase transformation efficiency, several different ecotypes (RLD, NO-O, ST0, and C24) of *Arabidopsis* were used for the transformation. Plants which were kanamycin resistant were screened for reduction in the level of LTP by Western analysis using an anti-*Arabidopsis* LTP antibody (chapter 3 of this dissertation). Over 50 independently transformed lines were screened by this method. Table 5-1 lists many of the transgenic lines and the relative reduction in LTP as determined by Western analysis. The plants with the greatest reduction appear, by qualitative measurement, to contain about 5-10% of the level of LTP as wild type plants and of plants transformed with pBI121 (Fig. 5-4). These plants, designated LTP4, LTP5, LTP12, and LTP18 were selected to

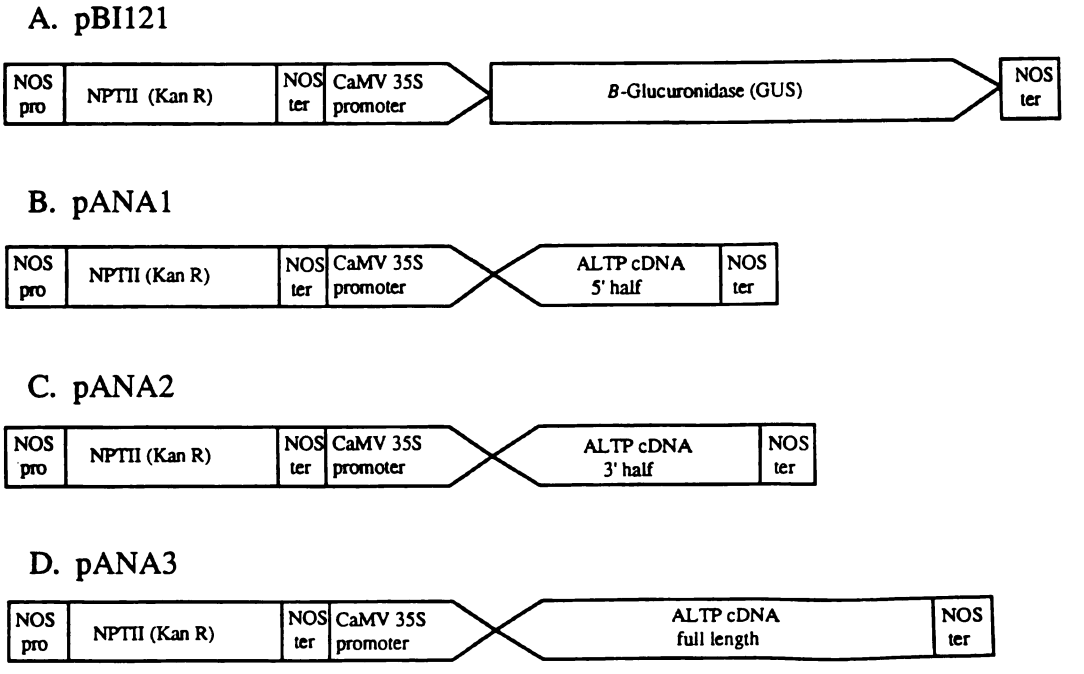


Figure 5-3. Structure of constructs used to produce transgenic plants.

Three antisense constructs were prepared; pANA1 (B), containing the 5' portion of the *Arabidopsis* LTP cDNA; pANA2 (C), containing the 3' portion of the cDNA; and pANA3 (D), containing the full length cDNA. Some plants were also transformed with pBI121 (A), as a negative control. The NPTII gene confers kanamycin resistance. Constructs were prepared by J. Botella.

Table 5-1. Antisense lines tested by Western analysis. This table represents plants which had a reduction in LTP in the primary screen and were later rescreened. There are approximately 30 lines which showed no reduction in LTP level in the primary screen, and these plants are not shown on this table. The relative level of reduction of LTP is shown by minus signs. (---) indicates that the level of protein was reduced to 5-10% of wild type levels, (--) indicates that the level of protein was reduced to 25-30% of wild type levels, (-) indicates that the level of protein was reduced to about 50% of wild type levels, and (0) indicates that there was no noticeable reduction in LTP levels in comparison to wild type plants.

Antisense line designation	Antisense construct	Relative level of LTP reduction	Parental ecotype
LTP1	pANA1	---	ST0
LTP2	pANA1	---	ST0
LTP3	pANA3	---	NO-0
LTP4	pANA2	---	RLD
LTP5	pANA3	---	RLD
LTP6	pANA3	--	RLD
LTP7	pANA3	--	RLD
LTP8	pANA3	-	RLD
LTP9	pANA3	--	RLD
LTP10	pANA3	--	RLD
LTP11	pANA3	0	RLD
LTP12	pANA3	---	RLD
LTP13	pANA2	0	RLD
LTP14	pANA1	--	RLD
LTP15	pANA1	--	RLD
LTP16	pANA1	0	RLD
LTP18	pANA3	---	RLD
LTP19	pANA3	-	RLD
LTP20	pANA3	--	RLD

Table 5-1 continued

Antisense line designation	Antisense construct	Relative level of LTP reduction	Parental ecotype
LTP22	pANA3	0	RLD
LTP26	pANA3	-	RLD
LTP30	pANA3	---	RLD
LTP31	pANA3	0	RLD
LTP34	pANA1	---	C24
LTP35	pANA1	---	C24
LTP36	pANA2	---	RLD
LTP39	pANA2	0	C24
LTP40	pANA2	0	C24
LTP41	pANA1	0	RLD
LTP42	pANA2	---	C24
LTP43	pANA2	0	C24

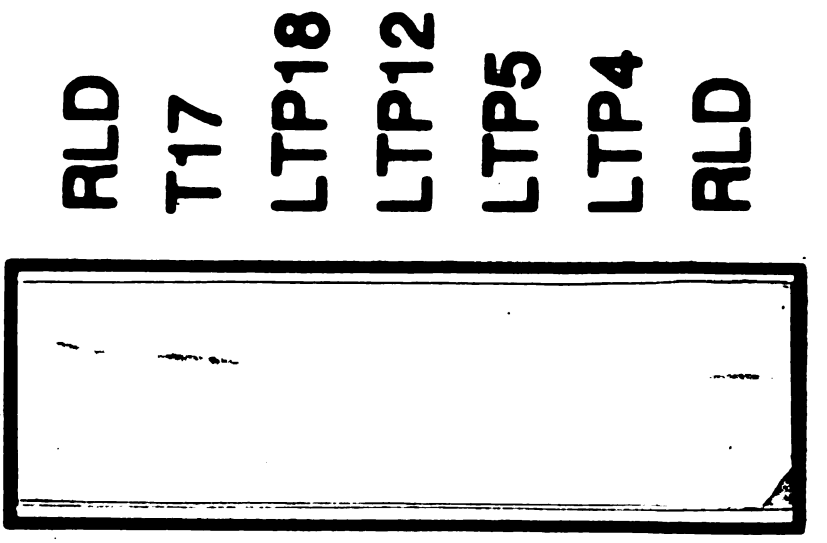


Figure 5-4. Western analysis comparing levels of LTP in wild type and transgenic plants.

Equal amounts of total soluble leaf protein were resolved by SDS-PAGE, transferred to nitrocellulose and immunodetected with anti-LTP antibodies. LTP4, LTP5, LTP12 and LTP18 represent plants which have been transformed with antisense LTP constructs. T17 represents a plant which has been transformed with pBI121. RLD is the wild type parent.

be homozygous for kanamycin resistance and thus for the antisense LTP construct. These lines were used for further analyses. All lines which were used for further analysis were in the RLD background. There appears to be no correlation with any particular antisense construct, and the level of reduction of LTP (Table 5-1). LTP4 contains the construct pANA2, which harbors the 3' half of the ALTP cDNA, and LTP5, LTP12, and LTP 18 contain pANA3, which includes the full length CDNA. Some plants containing pANA1, which harbors the 5' half of the cDNA, also have very low levels of LTP, but these plants were not in an RLD background. T17, which contains pBI121, but no antisense cDNA, had similar levels of LTP as wild type plants (Fig 5-4).

Phenotypic characterization of transgenic plants - If LTP played an essential role in the plant, one would expect that plants which had a greatly reduced level of the protein would exhibit some marked phenotypic changes with respect to control plants. To quantitate the growth rate of the plants, a growth curve measuring the fresh weight of the aerial portion of the plants was carried out. There was essentially no differences between the growth rates of control and antisense plants. LTP4, LTP5, and LTP18, were somewhat larger than the control plants, throughout the period growth was measured, but LTP12 was smaller (Fig. 5-5). This minor variation does not correlate with the amount of residual LTP in the antisense plants. Western analysis indicates that LTP4 has the greatest reduction in protein levels, while LTP5, LTP18, and LTP12 appear to have slightly higher

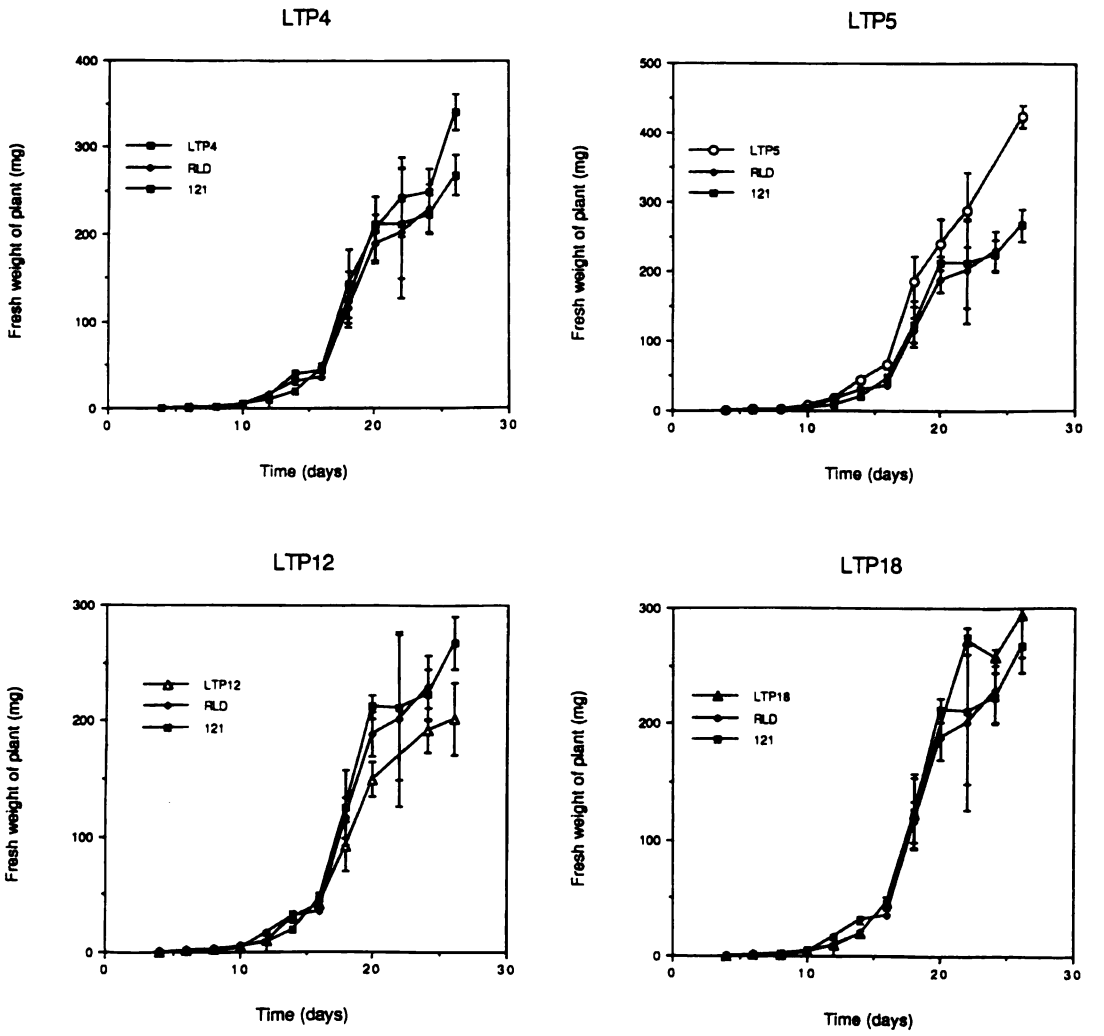


Figure 5-5. Growth of wild type and antisense plants.

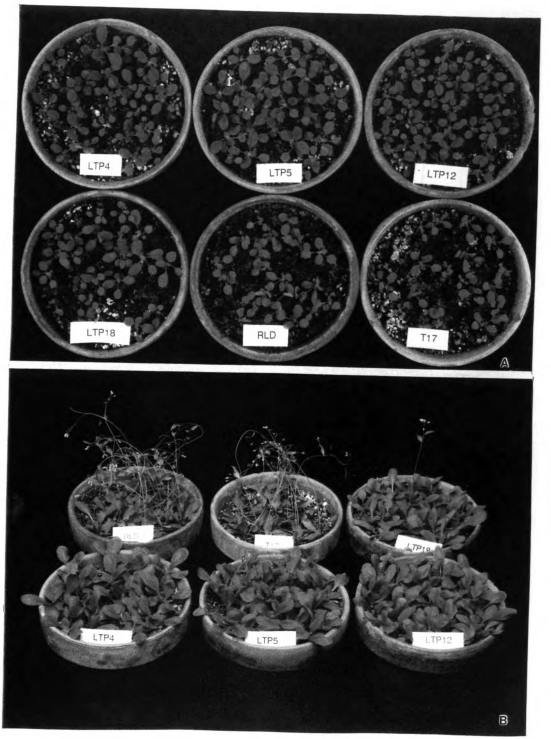
Means of measurements of fresh weight of the entire aerial portion of the plant (n=10) were determined. Each graph represents the growth of one antisense line compared to wild type plants and control plants containing a CaMV-GUS fusion (T17). Where no error bar (SE) is shown, the SE is less than the size of the symbol.

levels. During the first two weeks of growth, antisense and control plants exhibited no obvious morphological or developmental differences (Fig. 5-6A). At 14 days after germination, control plants started bolting, but flowering was delayed in the antisense plants (Fig. 5-6B, 5-7). LTP4, LTP5, and LTP18 started bolting 2-3 days after control plants, and bolt initiation was delayed for 7 days in LTP12 (Fig. 5-7). The germination rate of antisense and control plants was also tested by planting a known number of seed in a pot and counting the number of resulting plants (Fig. 5-8). Germination rates for the control plants were approximately 80%. The germination rates of antisense plants ranged from 60% to 90%. Again, there appeared to be no correlation between the amount of residual LTP in the antisense lines and germination rates.

To examine a possible role of LTP in cuticle formation, analysis of wax and cutin composition in wild type and transgenic plants was performed using gas chromatography. A comparison of chromatographs shows that there is no quantitative or qualitative differences in either the wax or cutin of the low LTP transgenic plants (Figs. 5-9, 5-10). The results of the analysis for LTP5 is presented here as it is representative of all transgenic lines. To test if the reduction in LTP had an effect on the structure of the epicuticular wax, the surface of the stems of wild type and antisense plants was examined by scanning electron microscopy (Fig. 5-11). The low levels of LTP appear to have no effect on the wax structure. Also, transmission microscopic analysis of the stem cuticular layer showed no obvious differences between wild type and antisense plants (Fig. 5-12).

Figure 5-6. Control and antisense plants at 15 and 25 days after planting.

A. Wild type, T17, and antisense plants at 15 days.
B. Wild type, T17, and antisense plants at 25 days.


LTP4
LTP5
LTP12
LTP18
RLD
T17
A
LTP4
LTP5
LTP12
B

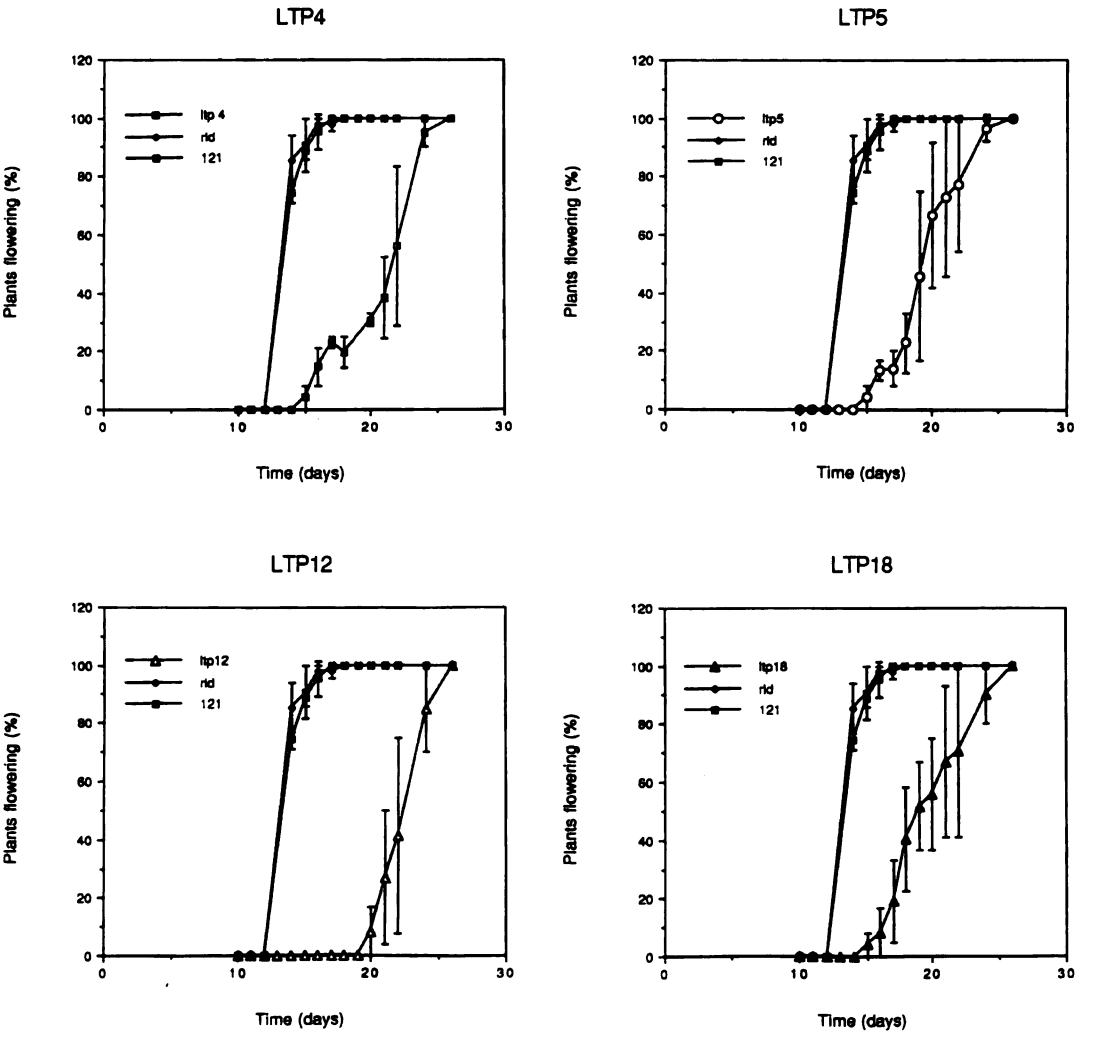


Figure 5-7. Time of flowering in control and antisense plants.

Control and antisense plants were analyzed for the time of flowering. Plants ($n \geq 10$) were observed from the time the first plant started flowering until all plants were flowering. Each graph represents the percentage of plants flowering of one antisense line as compared to the control plants. Where no error bar (SE) is shown, the SE is less than the size of the symbol.

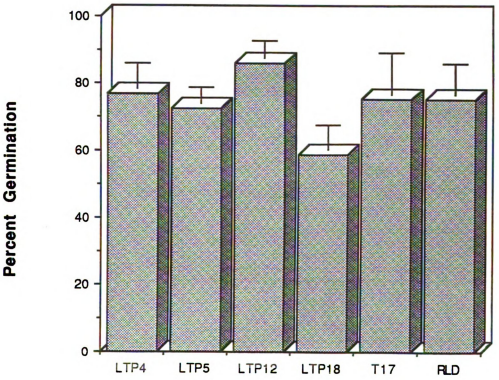


Figure 5-8. Percent germination of antisense and control plants.

A known number (n=32) of seed were planted, and the number of plants which resulted were counted.

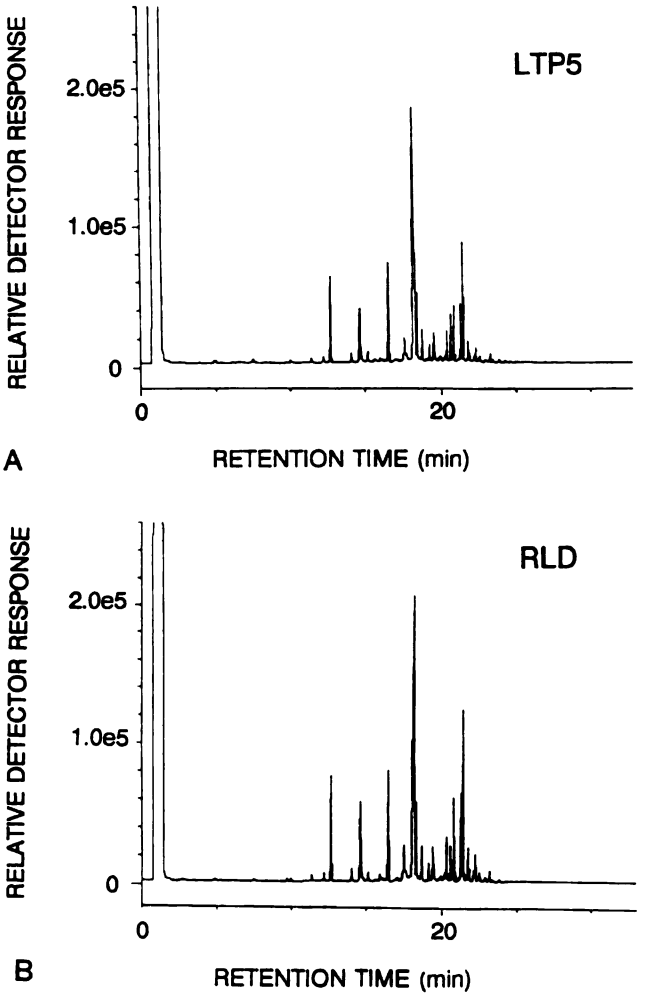


Figure 5-9. Gas chromatography analysis of wax composition.

Epicuticular wax was extracted from leaves of wild type (RLD) and antisense plants, and separated by gas chromatography on a 15 m X 0.53 mm ID Supelco SPB-1 column. The elution profile of LTP5 is representative of all antisense lines tested. This analysis was performed by U. Hecht.

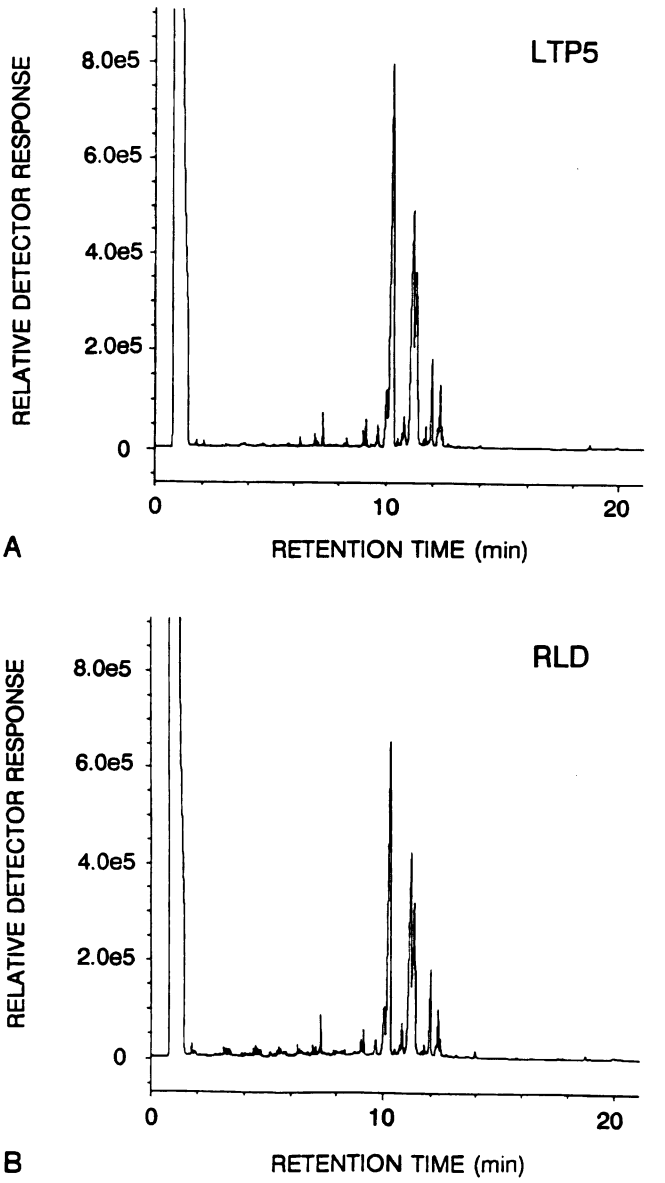


Figure 5-10. Gas chromatography analysis of cutin composition.

Cutin was extracted from leaf tissue of wild type (RLD) and antisense plants and separated by gas chromatography on a 15 m X 0.53 mm ID Supelco SPB-1 column. The elution profile of LTP5 is representative of all antisense lines tested. This analysis was performed by U. Hecht.

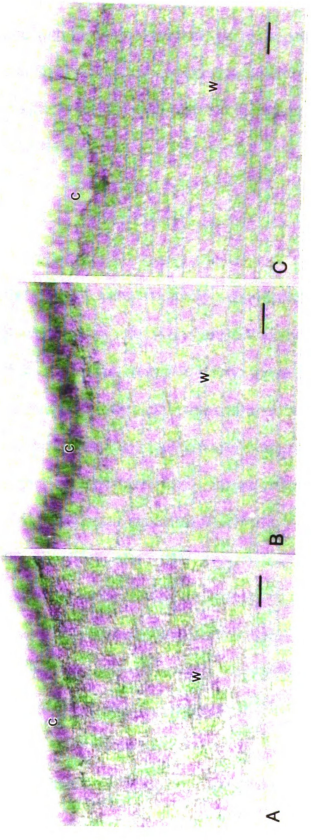
Figure 5-11. Scanning electron microscopy analysis of epicuticular wax of wild type and antisense stems.

Stem tissue of wild type (RLD) and antisense plants was frozen and viewed by cryo scanning electron microscopy.
A. LTP 5
B. LTP12
C. LTP18
D. RLD


B
D
A
C

Figure 5-12. Transmission electron microscopy analysis of the wax and cutin of wild type and antisense stems.

Stem tissue of wild type (RLD) and antisense plants was fixed, sectioned, and viewed with a transmission electron microscope. On average, 40 sections of each of 5 sample preparations were viewed.
A. LTP5
B. LTP12
C. RLD


A
B
C
w
w
w
c
c
c

To test the possible role of LTP in plant defense, control and antisense plants were infected with the *Arabidopsis* fungal pathogen, *Erysiphe cruciferae*. The results of this analysis were very ambiguous, as there was a variety of levels of fungal growth on the infected plants, with no correlation as to the presence or absence of an antisense construct.

DISCUSSION

Lipid transfer proteins were originally isolated based on their ability to transfer phospholipids between natural and artificial membranes *in vitro*. It has, however, been difficult to assign an *in vivo* role to these proteins. Non-specific LTPs have been isolated from several plant species, and recent evidence indicates that these proteins do not play a part in intracellular lipid transfer. A maize LTP was shown to be synthesized on membrane bound polysomes (Tchang, *et al.*, 1988; Vergnolle, *et al.*, 1988) and a spinach LTP was shown to be cotranslationally inserted into the ER (Bernhard, *et al.*, 1991; chapter 2 of this dissertation), thus indicating that it enters the secretory pathway. A barley LTP has been found in aleurone cell culture medium (Mundy and Rogers, 1986) and a carrot LTP has been found in carrot embryogenic cell culture medium (Sterk, *et al.*, 1991). More recently, an *Arabidopsis* LTP has been localized to the cell wall (Thoma, *et al.*, 1993; chapter 3 of this dissertation).

In addition to its extracellular location, LTP is synthesized in very specific

tissue and cell types. LTPs from carrot, maize, tobacco are found exclusively in the aerial portions of mature plants (Sterk, *et al.*, 1991; Sossountzov, *et al.*, 1991; Fleming, *et al.*, 1992), and an *Arabidopsis* LTP is found predominantly in the aerial parts of mature plants (Thoma, *et al.*, 1993; chapter 3 of this dissertation). Immunocytochemical analysis showed that an *Arabidopsis* LTP is localized mainly to epidermal cells (Thoma, *et al.*, 1993; chapter 3 of this dissertation), and *in situ* hybridization showed that expression of a tobacco LTP was confined mainly in epidermal cells of the aerial portion of plants and the L1 layer of the shoot meristem (Fleming, *et al.*, 1992). A second tobacco LTP is expressed predominantly in tapetal cells (Koltunow, *et al.*, 1990). Analysis of transgenic *Arabidopsis* containing an *Arabidopsis* LTP promoter-GUS fusion, showed that expression in adult plants is most prominent in stem and leaf epidermal cells, leaf vascular tissue, the stigma, floral nectaries, pollen grains, and at the base of lateral roots (chapter 4 of this dissertation).

Clearly, the extracellular location of the protein and its cell-type specific and tissue specific expression preclude a role for this protein in intracellular lipid transfer. In an effort to demonstrate an *in vivo* function for plant nsLTPs transgenic *Arabidopsis* plants that contain an *Arabidopsis* nsLTP cDNA in reverse orientation behind the CaMV 35S promoter have been produced. There have been numerous reports in which gene expression of transgenic plants has been inhibited by antisense RNA (Ecker and Davis, 1986; Smith, *et al.*, 1988; Van der Krol, *et al.*, 1988). The reduction of target gene expression using antisense techniques has

been useful to generate mutants for the discovery of the biochemical and biological function of target genes; for example, the role of the gene pTOM13 in ethylene biosynthesis was determined in this manner (Hamilton, *et al.*, 1990). *Arabidopsis* LTP appears to be encoded by at least two genes as shown by high stringency Southern analysis (chapter 4 of this dissertation). Assuming that these genes are similar enough that both would be impaired by this technique, I should be able to determine the function of the protein within the plant.

Antisense RNA techniques allowed us to produce plants which contained reduced levels of LTP. There were varying levels of reduction with the most dramatic containing no more than 10% of LTP as their wild type counterparts. If LTP has an essential function in a plant under normal growth conditions, one would expect to see an obvious alteration in phenotype of the antisense plants. There is essentially no consistent difference in the growth rates (accumulation of fresh weight) or germination rates between control and antisense plants. There is a delay in flowering in the antisense plants, but at this time, this phenotype cannot be reconciled with any possible role this protein may have. A more detailed analysis of the late flowering phenotype is necessary.

Based on their extracellular location, cell- and tissue-type specific localization and expression patterns (predominance in epidermal cells of aerial portions of plants), and ability to bind fatty acids, it has been proposed that LTPs may play a role in cuticle formation (Sterk, *et al.*, 1991; Thoma, *et al.*, 1993; chapter 3 of this thesis). To test this possible function for LTP, the wax and cutin

composition of antisense and wild type plants has been analyzed by gas chromatography. Comparison of the results shows that there is essentially no difference, quantitatively or qualitatively, in the wax or cutin of the antisense plants. Low temperature scanning electron microscopy was used to observe the structure of the wax on the stems of antisense and wild type plants and transmission electron microscopy was used to compare the cuticle. Again, no difference was observed between the transgenic and control plants. These data indicate that either LTP is not involved in cuticle formation or that the protein is still present in levels sufficient to carry out its role under normal growth conditions. As the cuticle is the major barrier to water loss in plants (Holloway, 1982), wax and cutin samples need to be extracted from plants which were grown under conditions, such as drought or salt stress, in which the cuticle becomes a more limiting component in plant survival.

The isolation of proteins which are homologous to LTPs and show antifungal and antibacterial activity *in vitro* (Terras, *et al.*, 1992; Garcia-Olmedo, personal communication), has led to a hypothesis that LTPs carry out a defense role *in vivo*. To test this hypothesis, antisense and control plants were inoculated with the *Arabidopsis* fungal pathogen, *Erysiphe cruciferae*. After one week, fungal growth on all plants was apparent, but there was no correlation between amount of fungal growth and the presence or absence of an antisense construct. Infection with *E. cruciferae*, an obligate pathogen, involves rubbing an infected leaf onto the leaf of a test plant. This method of inoculation is not quantitative, so if there is a

slight difference in susceptibility to this pathogen in the antisense plants, it will not be apparent. This type of test needs to be repeated using fungal or bacterial pathogens in which the amount of inoculum can be monitored more carefully.

The expression pattern of an *Arabidopsis* LTP - ß-glucuronidase (GUS) fusion in transgenic plants has led to the hypothesis that LTPs may be involved in phenylpropanoid metabolism (chapter 4 of this dissertation). To test this hypothesis, staining of stem sections could be carried out to determine if the lignin content of antisense plants has been altered. Lignin and phenolic acid content of antisense plants could also be determined by gas chromatographic analysis (C. Chapple, personal communication). Results of a quantitative pathogenesis experiment may be helpful here, as phenylpropanoid compounds are induced upon pathogen infection (Dixon and Lamb, 1990). Again, a limiting factor to any of these experiments is the presence of two LTP genes in *Arabidopsis* and the fact that the low level of protein left in these plants may be enough to carry out the its role.

In conclusion, the role of LTP within a plant is still unknown. Possibly, further analysis of the antisense plants described in this paper will provide an answer.

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CHAPTER 6

SUMMARY AND CONCLUSION

Lipid transfer proteins were first isolated based on their ability to enhance the rate of lipid transfer between natural and artificial membranes *in vitro*. It has been a long held assumption that these proteins have a similar activity *in vivo*, but there has been no experimental evidence to support this supposition. My research began as an attempt to examine the role of plant nsLTPs in membrane biogenesis and to use existing technologies to demonstrate their role in this process *in vivo*. However, as my research progressed, it became clear that plant nsLTPs were not directly involved in intracellular lipid transfer, and I began a search for an alternate function. This chapter is a summary of the evidence which demonstrated that nsLTPs are not actually involved in lipid transfer (chapters 2 and 3 of this dissertation), the experimental observations which led to the formation of hypotheses about the role of these proteins (chapters 3 and 4 of this dissertation), the results of the experiments carried out to test these hypotheses (including chapter 5 of this dissertation), and suggestions on future research.

The proposed mechanism of LTP mediated phospholipid transfer involves the extraction of a lipid molecule from the cytoplasmic face of the ER (or other biosynthetic membrane such as the chloroplast), movement of the lipid-protein complex through the cytosol, and release of the lipid molecule to the outer leaflet of the target membrane. The LTP would then be able to extract a lipid from the target membrane (in the case of the exchange process) or be free to diffuse back to the biosynthetic membrane to bind another lipid (in the case of net transfer). Such a mechanism of action implies that LTPs are cytosolic, and there are

numerous reports which state that LTPs are cytoplasmic proteins (Arondel and Kader, 1990; Helmkamp, 1990). These assertions are based on the fact that LTPs are soluble proteins and that a direct role in intracellular lipid transfer obviates the need for a cytoplasmic location. However, there is no experimental evidence to support such statements.

There is much recent evidence which demonstrates that plant nsLTPs are, in fact, extracytoplasmic. Comparison of the deduced amino acid sequence of a spinach LTP cDNA (Bernhard, *et al.*, 1991; chapter 2 of this dissertation) with a directly determined amino acid sequence (Boullion, *et al.*, 1987) revealed the presence of a 26 amino acid N-terminal extension. The extension had all the properties of a signal peptide which directs proteins into the ER (von Heijne, 1985): (1) an amino terminal location, (2) 20-40 amino acids in length, (3) Ala or other small, uncharged residue in positions -1 and -3 from the cleavage site, (4) a helix breaking residue such as Pro at the -5 position, and (5) a stretch of 8-10 small, uncharged residues N-terminal to the helix breaking residue. The presence of a putative signal peptide was very surprising considering the supposed cytosolic location and function of LTPs. Proteins which enter the secretory system are either secreted or become localized within a compartment of the endomembrane system, e.g., the ER, the Golgi apparatus, or the vacuole (Bednarek and Raikhel, 1992).

In vitro transcription and translation in the presence of microsomal membranes and protease protection experiments demonstrated that the extension

was a functional signal peptide directing the spinach LTP into the ER (Bernhard, *et al.*, 1991; chapter 2 of this thesis). This supports the observation that a maize LTP is synthesized on membrane bound polysomes (Vergnolle, *et al.*, 1988). It has subsequently been shown that a barley LTP (Barley I) also contains a signal peptide (Madrid, 1991), and sequence analysis shows that carrot (Sterk, *et al.*, 1991), castor (Tsuboi, *et al.*, 1991), tobacco (Fleming, *et al.*, 1992), tomato (Torres-Schumann, *et al.*, 1992), wheat (Dieryck, *et al.*, 1992), and *Arabidopsis* (chapter 5 of this dissertation) contain similar N-terminal sequences.

The entrance of plant nsLTPs into the secretory pathway is inconsistent with the proposed role of the protein in intracellular lipid transfer. To determine the precise location of the protein, and to resolve the possibility that the protein may move from the ER to the cytoplasm by some novel mechanism, I produced antibodies against an *Arabidopsis* LTP:protein A fusion and localized the protein at the ultrastructural level using immunoelectron microscopy (chapter 3 of this dissertation; Thoma, *et al.*, 1993). The protein was found exclusively in the cell wall, and it was most concentrated in epidermal cells. The cell wall localization was consistent with other accounts reporting that plant nsLTPs were secreted. A barley protein which was secreted in aleurone cell culture media (Mundy and Rogers, 1986), was shown to contain considerable homology to nsLTPs (Bernhard and Somerville, 1989) and to stimulate PC transfer from microsomes to potato mitochondria in *in vitro* assays (Breu, *et al.*, 1990). It has also been shown that a carrot LTP is secreted by carrot embryogenic cell cultures (Sterk, *et al.*, 1991).

As all of this evidence pointing to an extracellular location accumulated, and it became apparent that plant nsLTPs could not be directly involved in intracellular lipid transfer or membrane biogenesis, it became imperative to try to determine the actual function of these proteins. The next part of this chapter will discuss suggested roles that LTP may have within a plant, and evidence which led to such suggestions. Finally, recommendations on future research directions will be discussed.

Glyceride synthesis

Lipid metabolism in developing seeds consists mainly of triacylglycerol (TAG) synthesis. TAGs serve primarily as a storage form of carbon in developing seeds and rapid synthesis of lipids has been observed during the period of seed weight gain (Murphy, *et al.*, 1989). TAG synthesis and modification occurs in the ER and these lipids are ultimately stored in oil bodies in the mature seed (Stymne and Stobart, 1987).

The occurrence of nsLTP in developing maize and castor seeds (Grosbois, *et al.*,1989; Yamada, *et al.*, 1990) and its expression in aleurone tissue (Skriver, *et al.*, 1992) led to a hypothesis that the protein may act as an acyl carrier in TAG synthesis (Yamada, *et al.*, 1990). This hypothesis, however, is inconsistent with the presence of LTP in non-lipid storing tissues. It is also inconsistent with the cell wall localization of the protein.

Fatty acid degradation

Upon germination of many seedlings, breakdown of reserve triglycerides begins and its products support seedling growth. Their degradation proceeds mainly by oxidation in the ß-position to the carboxyl group and sequential removal of carbon units. The process of ß-oxidation occurs in the glyoxosomes where it is coupled to the glyoxolate cycle. This allows for the conversion of the carbon to isocitrate which can eventually be shuttled to the cytoplasm and converted to sucrose (Andrews and Ohlrogge, 1990).

Immunoelectron microscopy has demonstrated that castor nsLTPA is localized to the glyoxysomes and secondary cell wall of xylem vessels in castor bean cotyledons (Tsuboi, *et al.*, 1992). This partial localization to the glyoxysomes plus the demonstration that nsLTPA could bind oleic acid and oleoyl CoA (Tsuboi, *et al.*, 1992) led to the hypothesis that this nsLTP could function as an acyl carrier in ß-oxidation. It has also been proposed that nsLTPA could function to enhance the activity of acyl CoA oxidase, the rate limiting enzyme in ß-oxidation, as it was observed that the presence of nsLTPA increased enzyme activity in an *in vitro* assay (Tsuboi, *et al.*, 1992).

However, there are some uncertainties regarding this hypothesis. First, subcellular fractionation showed that only 13% of the nsLTPA was found in the glyoxysomal fraction, and the major portion was found in the soluble fraction (Tsuboi, *et al.*, 1992). Proteins which are targeted to glyoxysomes or peroxisomes are synthesized in the cytosol and are post-translationally transported into the

organelle. Thus, it is suggested that at least part of the protein associated with the soluble fraction represents cytosolic protein. Secondly, signals which direct proteins into glyoxysomes are generally a small sequence found on the C-terminal portion of the protein (Gould, *et al.*, 1989), and there is no such sequence found in castor nsLTPA. Tsuboi and coworkers also show evidence that castor nsLTPA is processed to a mature form in the presence of purified glyoxysomes. Import of proteins into peroxisomes or glyoxysomes is not generally associated with the removal of a presequence or with any other modification of the imported protein (Gould, *et al.*, 1989). There have been exceptions to this rule, for example a watermelon glyoxysomal malate dehydrogenase is synthesized with an amino-terminal transit peptide (Gietl, 1990). This transit peptide, however, bears no resemblance the N-terminus of castor nsLTPA. The castor nsLTPA N-terminal signal (Tsuboi, *et al.*, 1991) has features representative of a signal peptide directing proteins to the ER (von Heijne, 1985). This is more consistent with the co-localization of the protein to vessel cell walls, than with the localization of the protein to the glyoxysomes. It is a possibility that the glyoxysome preparations were contaminated with ER. In addition, the antibodies used for the localization were not affinity purified, which could lead to artifacts. The discrepancy between the localization of the protein to two locations which require different targeting mechanisms, and the lack of a peroxisomal/glyoxysomal targeting signal on the castor LTP, allow for reservations concerning the role of this protein in fatty acid degradation.

Cuticle formation

The cuticle is a continuous layer of predominantly lipophilic material found on the outermost surface of the aerial portions of plants (Holloway, 1982). A major component of the cuticle is cutin, an insoluble polymer composed mainly of C_{16} and C_{18} hydroxylated and epoxygenated fatty acids (Kolattukudy, 1982). Another component of the cuticle is wax. Plant epicuticular waxes are generally composed of a diverse mixture of long chain hydrocarbons, including long chain (C_{12}-C_{36}) fatty acids (Kolattukudy, 1982). The aliphatic components of the cuticle are synthesized in the epidermal cells and are delivered to the outside of the cell where it is thought that esterification takes place (Croteau and Kolattukudy, 1974). The mechanism by which the monomers are delivered to the outside of the cell is unknown. It is possible that nsLTPs could bind the aliphatic monomers, carry them through the aqueous environment of the cell wall, releasing them once they have transversed the wall.

Tissue-type, cell-type, and subcellular localization of an *Arabidopsis* LTP (chapter 3 of this dissertation; Thoma, *et al.*, 1993) and expression patterns of the carrot, maize, and tobacco LTP genes (Sterk, *et al.*, 1991; Sossountzov, *et al.*, 1991; Fleming, *et al.*, 1992), are all consistent with a hypothesized role for LTP in cuticle formation.

Immunoblot analysis demonstrated that *Arabidopsis* LTP is mainly present in the aerial portions of the plant; flowers, siliques, stems, petioles, and leaves, very little LTP present in root tissue. Immunocytochemical localization shows that

the *Arabidopsis* LTP is present mainly in the cell walls of epidermal cells, the cell type in which the synthesis of cuticle monomers occurs. LTP is seen to be particularly concentrated in guard cells. This is also consistent with a role in cuticle formation. Normally, epidermal cells contain a cuticle layer only on their outer surface. Guard cells, however, are covered with a thick cuticle that not only extends over the outer surface, but also the surfaces facing the substomatal chamber and the stomal pore (Esau, 1977). LTP is also heavily concentrated in the cells walls surrounding cells at or near the stigma surface. The epidermal and subepidermal layers of a stigma can produce a secretion containing lipid components and phenolic compounds (Martin and Brewbaker, 1971) and it is possible that LTP could play a role in the deposition of the lipid components of the secretion. There is a small amount of LTP associated with the epidermal cells of root tissue and LTP promoter-GUS fusions are active at the base of lateral roots (chapter 4 of this dissertation). This is inconsistent with a role for LTP in cuticle formation as roots are not generally thought to contain cutin.

RNA gel blot analysis shows that the carrot LTP is expressed in embryogenic cell cultures, the shoot apex of seedlings, developing flowers, and maturing seed (Sterk, *et al.*, 1991). A tobacco LTP has been shown to be expressed in the aerial parts of plants, primarily in epidermal cells and in the L1 layer of the shoot meristem (Fleming, *et al.*, 1992). A maize LTP is highly expressed in the epidermal cells of the aerial portions of maize seedlings (Sossountzov, *et al.*, 1991). *In situ* hybridization patterns show that the carrot LTP

mRNA is present in the protoderm (a cell layer which gives rise to the epidermis) of somatic and zygotic embryos, in the tunica (top cell layer(s)) of the shoot apical meristem, transiently in the epidermis of leaf primordia and floral organs, and in the pericarp epidermis, seed coat, and outer epidermis of the integument in maturing seeds (Sterk, *et al.*, 1991). As with the *Arabidopsis* LTP, the known locations of cuticle formation correspond well with the locations in which the carrot LTP gene is expressed. It has been suggested (Sterk, *et al.*, 1991) that the presence of LTP in embryos may represent a mechanism to slow water uptake in a hypotonic environment, thus restricting unlimited and potentially destructive cell expansion.

To test the role of LTP in cuticle formation, *Arabidopsis* plants containing a greatly reduced level of LTP due to the introduction of an *Arabidopsis* LTP in reverse orientation between the CaMV 35S promoter and nos terminator were produced (chapter 5 of this dissertation). Gas chromatographic analysis revealed no qualitative or quantitative differences in the epicuticular wax or cutin composition between antisense and wild type plants. Also electron microscopic analysis revealed no differences in the wax or cutin structure (chapter 5 of this dissertation). These data suggest that LTP is not involved in cuticle formation. However, the negative results achieved by the antisense approach are not conclusive. I was unable to obtain any plants that were completely devoid of LTP. Thus, although the level of LTP in the plants used for analysis was greatly reduced, it could be present at levels sufficient to carry out its function under normal growth conditions. Also, it has been demonstrated that there are at least two closely

related LTP genes in *Arabidopsis* (chapter 4 of this dissertation). The antisense approach may not have been successful in reducing the expression of both genes. Future experiments include analysis of wax and cutin composition of plants grown under stress conditions where the cuticle is essential for the survival and/or growth of the plant.

Protection against pathogen attack

Recently, a basic, 9 kD protein, which is homologous to nsLTPs, has been isolated from radish seeds and shows antifungal activity *in vitro* (Terras, *et al.*, 1992). Also, proteins homologous to LTPs, which strongly inhibit bacterial and fungal pathogens in *in vitro* assays, have been isolated from etiolated barley and maize leaves. (F. Garcia-Olmedo, personal communication).

In addition, thionins, which have toxic effects on bacteria, fungi, and insects (Bohlman and Apel, 1991), have several features in common with plant LTPs; they are low molecular weight, basic proteins with several conserved cysteine residues. A barley leaf thionin is found in the cell wall, with the highest concentration in the outer wall of the epidermal cell layer (Bohlman, *et al.*, 1988). This localization is similar to that of *Arabidopsis* LTP in all green tissue examined.

In an attempt to determine whether LTPs had an antifungal activity *in vivo*, we infected wild type and antisense plants with the *Arabidopsis* fungal pathogen, *Erysiphe cruciferae*. The manner of infection involves rubbing an infected leaf onto a test leaf, which gave ambiguous, non quantitative results. This type of test bears

repeating using a system which will allow for more controlled and quantitative testing.

Phenylpropanoid metabolism

The analysis of transgenic plants containing an *Arabidopsis* LTP promoter-GUS fusion showed that the promoter was active in developing *Arabidopsis* seedlings and the pattern of expression observed in cotyledons and primary leaves generally followed that of xylem differentiation. In adult plants, expression was found mainly in vascular tissue or cell types covered with lipophilic substances (chapter 4 of this dissertation). Lignin, the structural polymer of xylem, and phenolic acids, a component of cuticular type substances, are both products of the phenylpropanoid pathway. In addition, analysis of the promoter region of the ALTP1 gene revealed the presence of several sequence elements commonly found in the promoters of biosynthetic genes of the phenylpropanoid biosynthetic pathway (Cramer, *et al.*, 1989; Lois, *et al.*, 1989; Ohl, *et al.*, 1990). These data suggest that LTP may have a role in phenylpropanoid metabolism, or that LTP genes are regulated in a manner similar to genes encoding enzymes of the phenylpropanoid biosynthetic pathway.

Phenylpropanoids are a class of plant natural products derived from phenylalanine. These compounds play important roles in plant development and in protection against environmental stress. For instance, flavonoids are pigments and UV protectants in epidermal cells, lignin is the major structural component in

xylem cell walls, and suberin is a lipophilic substance commonly found in the casparian strip of the endodermis (Esau, 1977). The induction of lignin in wheat (Moerschbacher, *et al.*, 1990), suberin deposition in potato (Roberts and Kolattukudy, 1989), and the accumulation of phenylpropanoid derived phytoalexins help protect a plant against mechanical damage and microbial attack (Dixon and Lamb, 1990).

When plants containing the ALTP1 promoter-GUS fusion were stained with X-Gluc 48 hours after infection with *E. cruciferae*, several regions of staining occurred. Examination of this tissue showed that each area was associated with tissue damage (chapter 4 of this dissertation). At this point it is not known whether this is due to hyphal growth into the tissue or mechanical damage due to the wounding procedure. The experiment should be repeated using other fungal and bacteria pathogens with different methods of inoculation.

In future research, the induction of LTP under different biotic and abiotic stresses needs to be examined. A tomato nsLTP is greatly induced in stems by NaCl, mannitol treatment, and ABA treatment (Torres-Schumann, *et al.*, 1992). Also, a small, basic barley protein, which has nearly 50% sequence identity with maize LTP, was shown to be drought induced (Plant, *et al.*, 1991). The production of phenylalanine ammonia-lyase (PAL), an enzyme in the phenylpropanoid pathway which contains similar sequence elements with the *Arabidopsis* LTP1 gene, is induced by wounding, light, and $HgCl_2$ treatment. Transgenic *Arabidopsis* containing LTP promoter-GUS fusions are the ideal system to test for stress

induction of LTPs, and the transgenic *Arabidopsis* containing an LTP antisense gene will be useful in testing the role of LTP in such stress responses *in vivo*.

In conclusion, the role of non-specific lipid transfer proteins in plants is still unknown. Their extracellular location and cell-type and tissue-type specific expression, precludes any direct role for these proteins in lipid transfer, but has provides us with other hypotheses concerning the role of this protein *in vivo*. Transgenic *Arabidopsis* containing reduced levels of LTP will provide us a tool with which to test my hypotheses, and hopefully elucidate the role of plant non-specific lipid transfer proteins.

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