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dissertation entitled

CHARACTERIZATION OF ACYL CARRIER PROTEIN GENES FROM ARABIDOPSIS THALIANA (L.) HEYNH. VAR. COLUMBIA

presented by

ALENKA HLOUSEK-RADOJCIC

has been accepted towards fulfillment
of the requirements for

Ph. D. degree in DEPT. OF BOTANY AND PLANT PATHOLOGY

Major professor

Date 7/25/92

CHARACTERIZATION OF ACYL CARRIER PROTEIN GENES FROM *ARABIDOPSIS THALIANA* (L.) HEYNH. var. COLUMBIA

by

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DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Botany and Plant Pathology

1992

ABSTRACT

CHARACTERIZATION OF ACYL CARRIER PROTEIN GENES FROM *ARABIDOPSIS THALIANA* (L.) HEYNH. VAR. COLUMBIA

By

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Multiple ACP isoforms have been characterized from *Arabidopsis thaliana* (L.) Heynh. var. Columbia. Immunoblot analysis and radiolabeling of ACPs via acylation of ACPs in crude protein extracts revealed the presence of a minimum of one tissue-specific and at least three constitutively expressed ACP isoforms in *Arabidopsis* leaves, roots, and developing and dry seeds.

Two ACP clones, *ACP1* and *ACP2* have been isolated and characterized from *Arabidopsis* genomic and cDNA libraries, respectively. The genomic clone *ACP1* encodes a 137 amino acids long preACP-1, having a 54 amino acids long transit peptide. Based on comparisons with the *Brassica campestris* cDNA ACP clone, *Arabidopsis ACP1* has three introns. Intron I is located in the transit peptide coding region, whereas introns II and III are located in the coding region for mature ACP-1 between amino acids 1 and 2 and 42 and 43, respectively. The *Arabidopsis ACP2* cDNA clone encodes a protein with a 52 amino acid transit peptide and an 83 amino acid mature protein. *ACP1* and *ACP2* have 70% identity at both nucleic acid and amino acid levels. DNA blot analysis indicated that *Arabidopsis* has a minimum of three ACP genes. Based on RNA blot analysis, the *ACP1* and *ACP2* genes are expressed

in leaves, roots, and developing seeds.

Expressing coding regions for mature ACPs in *Escherichia coli*, three *Arabidopsis* ACP isoforms were partially purified on DE-52 columns and identified by immunoblotting with antiserum to spinach ACP-I. Even the *Arabidopsis* ACP-2 and ACP-3 isoforms that differ in a single amino acid, were resolved by native and denaturing 1M urea PAGE. ACP-1, ACP-2 and ACP-3 were found to comigrate with different *Arabidopsis* ACP isoforms found in leaves, roots, and seeds, suggesting that all three of them are constitutively expressed ACP isoforms.

To peace.

ACKNOWLEDGMENTS

I would like to thank to Dr. J.B. Ohlrogge for having made my work and stay exciting. His support, advice and incredible amount of patience and understanding were invaluable. Thanks to all the members of the Ohlrogge's lab from the past and present; their support made my life so much easier. Very special thanks to Dusty, Jan, Katherine and Paul, who were with me even when the exciting results were replaced by big disappointment.

My thanks also go to the members of my guidance committee, Drs. Barbara Sears, William Smith and Chris Somerville for their continuous support during my stay as a graduate student. Thanks to Chris Somerville for giving me the opportunity to feel the excitement of being briefly a part of his group.

I would like to thank my parents, Emilija and Zdenko, and to my brother, Zvonimir, for their love and long-distance support. I would like to give a special thanks to Mirna for the wittiness and joy, and to Alan for his smiles and relatively peaceful nights. Finally, my greatest appreciation goes to Zlatko for his constant love and encouragement.

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LIST OF ABBREVIATIONS

amp	ampicillin
BCIP	bromochloroindolyl phosphate
Blotto	5% (w/v) nonfat dry milk (Carnation Co., Los Angeles, CA) in TBST
5x Denhardt's	0.1% (w/v) bovine serum albumin, 0.1% (w/v) PVP, 0.1 % (w/v) Ficoll 6000
DEPC	diethyl pyrocarbonate
dH_2O	distilled water
ddH_2O	double distilled water
DTT	dithiothreitol
EDTA	(ethylenedinitrilo)- N,N,N',N'-tetraacetic acid
EGTA	ethylene glycol-bis(b-aminoethyl ether)-N,N,N',N'-tetraacetic acid
GUS	b-glucuronidase
IPTG	isopropyl-b-D-thiogalactopyranoside
LB.....	10 g Bacto-tryptone (Difco), 5 g yeast extract (Difco), 5 g NaCl in 1 l dH_2O
LB plates	LB broth with 10 g Bacto-agar (Difco)
MES	2-(N-Morpholino)ethanesulfonic acid
MOPS	(3-[N-Morpholino]propanesulfonic acid
NBT	nitroblue tetrazolium
NEM	N-ethylmaleimide
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction

PEG	**polyethylene glycol**
PVP	**polyvinylpyrrolidone**
SDS	**sodium dodecyl sulphate**
SM buffer	100 mM NaCl, 10 mM $MgSO_4$, 50 mM Tris HCl, pH 7.5, 0.01 % gelatin
1x SSPE	0.15 M NaCl, 0.01 M NaH_2PO_4, 0.001 M EDTA, disodium salt, pH 7.4.
TBST	10 mM Tris HCl, pH 8.0, 150 mM NaCl, 0.05% (v/v) Tween 20
TCA	**trichloroacetic acid**
TE	10 mM Tris HCl, pH 7.4, 1 mM EDTA
TEA-Ac	**triethylammonium acetate**

CHAPTER ONE

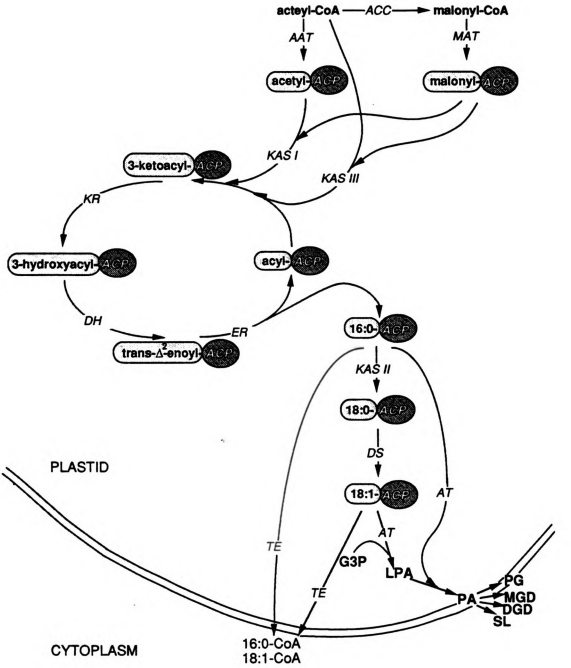
INTRODUCTION

Fatty acid synthesis

Fatty acid biosynthesis occurs through the sequential elongation of acyl chains with two carbon units (52). The initial reactions in fatty acid biosynthesis start with a transfer of a malonyl group to the phosphopantetheine arm of acyl carrier protein (ACP), followed by a condensation reaction with acetyl-CoA (22). The four carbon acetoacetyl moiety, while still bound to ACP, goes through ketoreduction, dehydration and enoyl reduction steps (52). For the synthesis of 16 or 18 carbon fatty acids this four step cycle is repeated seven or eight times, respectively (Fig.1). Acyl carrier protein, via the thioester bond that is formed between the carboxyl group of the acyl chain and the sulfhydryl group of the phosphopantetheine (Fig. 2), "activates" the acyl group and carries it from one catalytic domain to the other, thereby functioning as a cofactor in fatty acid biosynthesis (30).

Although the set of reactions in fatty acid biosynthesis is similar in all living organisms, the molecular organization of fatty acid synthases differs (56). In animals, fatty acid synthase is a multifunctional protein (referred to as type I) which is composed of seven catalytic

Figure 1. Schematic representation of fatty acid and some steps of lipid biosynthesis in higher plants. AAT - acetyl-CoA:ACP transacylase; ACC - acetyl-CoA carboxylase; ACP - acyl carrier protein; AT - acyltransferase; DH - β-hydroxyacyl-ACP dehydrase; DGD - digalactosyl diacylglycerol; DS - stearoyl-ACP desaturase; ER - enoyl-ACP reductase; G3P - glycerol-3-phosphate; KAS I - β-ketoacyl-ACP synthase I; KAS II - β-ketoacyl-ACP synthase II; KAS III - β-ketoacyl-ACP synthase III; KR - β-ketoacyl-ACP reductase; LPA - lysophosphatidic acid; MAT - malonyl-CoA:ACP transacylase; MGD - monogalactosyl diacylglycerol; PA - phosphatidic acid; PG - phosphatidylglycerol; SL - sulphoquinovosyl diacylglycerol; TE - thioesterase; 16:0-CoA - palmitoyl-CoA; 18:0-CoA - stearoyl-CoA;


acteyl-CoA
ACC
malonyl-CoA
AAT
MAT
acetyl-ACP
malonyl-ACP
KAS I
KAS III
3-ketoacyl-ACP
KR
3-hydroxyacyl-ACP
acyl-ACP
DH
ER
trans-Δ-enoyl-ACP
16:0-ACP
KAS II
18:0-ACP
DS
PLASTID
18:1-ACP
AT
AT
TE
G3P
LPA
TE
PA
PG
MGD
DGD
SL
CYTOPLASM
16:0-CoA
18:1-CoA

Prosthetic group structure of acyl carrier protein

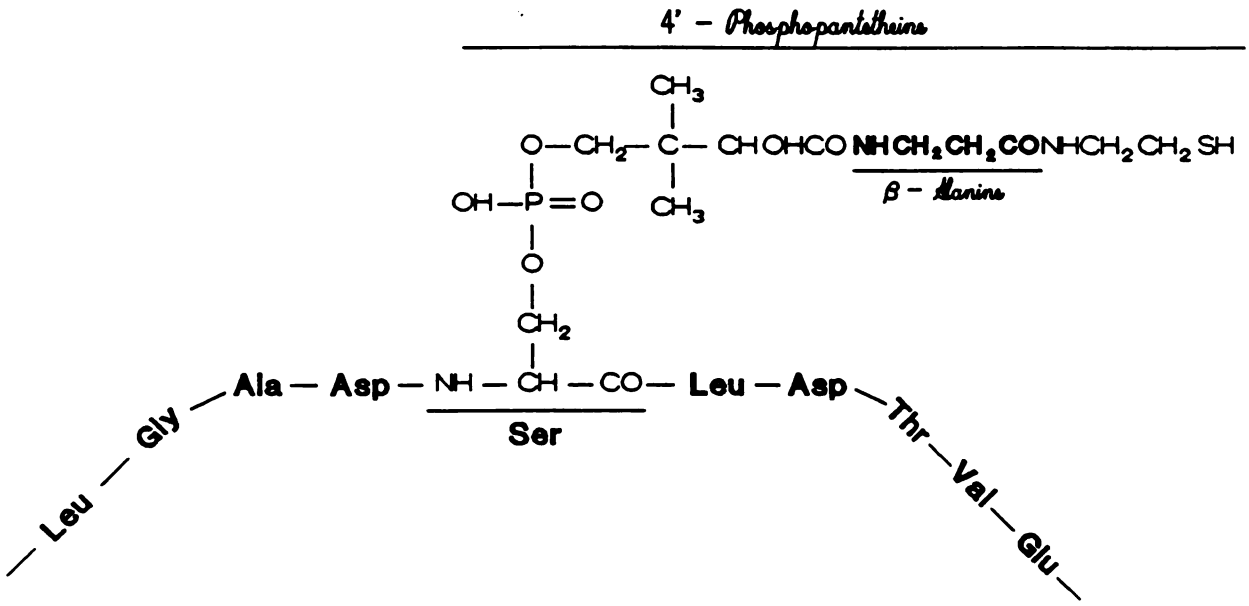


Figure 2. Prosthetic group structure of acyl carrier protein. 4'-phosphopantetheine group is bound to a serine residue in the middle of the mature ACP.

domains and a domain for ACP. The functional enzyme is present as a homodimer. Yeast also has a type I organization, but the FAS is composed of two subunits, α and β. The α subunit contains ACP, β-ketoacyl reductase and β-ketoacyl synthase domains, whereas the β subunit contains domains for the remaining five enzymes: the acetyl transacylase, the enoyl reductase, the dehydratase and the malonyl/palmitoyl transacylase. In plants and most prokaryotes fatty acid synthase is of the nonassociated type (type II). Reactions are catalyzed by six separate enzymes and the protein cofactor, ACP (30). The functional organization of the enzymes *in vivo* is unknown.

Functions of ACP in lipid biosynthesis

The principal products of *de novo* fatty acid biosynthesis in plants are palmitic and stearic acids (52). However, plant membranes are very rich in mono- and polyunsaturated fatty acids (58). Therefore, the majority of palmitic and stearic acids undergo further modifications, some of which are ACP mediated. McKeon & Stumpf (28) reported that stearoyl-ACP desaturase, which is involved in the biosynthesis of oleic acid, prefers stearoyl-ACP over stearoyl-CoA as a substrate. The enzyme is reportedly found in a soluble fraction (47). Partially purified stearoyl-ACP desaturase requires soluble ferredoxin, ferredoxin NADPH reductase and molecular oxygen (28). Based on all these characteristics, the stearoyl-ACP desaturase is considered to be localized in the chloroplast stroma.

In addition to the biosynthesis of fatty acids, plastids are also the site of biosynthesis of some plastidial membrane glycerolipids (41). However, only a fraction of acyl groups synthesized in plastids are used as substrates in the plastidial reactions of glycerolipid biosynthesis. The remaining portion is transported into the extraplastidial compartment to enter the cytoplasmic pathway of lipid biosynthesis. Acyl-ACPs entering the plastidial pathway of glycerolipid biosynthesis are substrates of two acyltransferases. The glycerol-3-phosphate acyltransferase is soluble (5), whereas monoacyl glycerol-3-phosphate acyltransferase is localized in the inner chloroplast membrane (1). In contrast to cytoplasmic acyltransferases, both plastidial acyltransferases prefer acyl-ACPs over the acyl-CoA substrates. In addition, the plastidial acyltransferases also have different affinities for acyl groups bound to ACP. The acyltransferase that transfers the acyl chain to the first position of the glycerol backbone prefers oleoyl-ACP, whereas the second transferase, which is responsible for the acylation of the second carbon on the glycerol backbone, favors palmitoyl-ACP (9). As a result of those specificities, plastidial glycerolipids have a distinct positional distribution of fatty acids when compared with cytoplasmatically synthesized glycerolipids (41).

Acyl groups entering the cytoplasmic pathway of lipid biosynthesis must be transported through the plastid envelope. This process starts with the hydrolysis of the acyl group from ACP, presumably followed by the transport of free fatty acid into the cytoplasm. On the outer plastid envelope membrane, acyl-CoA synthase esterifies free fatty acids to CoA,

providing substrates for extraplastidial lipid biosynthesis. Hydrolysis of acyl-ACPs occurs in plastids by the action of an acyl-ACP hydrolase (thioesterase) (52). Partial purification of the enzyme from avocado mesocarp revealed the presence of a hydrolase that in addition to a preferential use of acyl-ACPs, showed great selectivity toward the chain length and the level of desaturation. Kinetic studies indicated that the preferred substrate was oleoyl-ACP (32).

Functions of ACP other than in fatty acid biosynthesis

In addition to the central position of ACP in fatty acid and lipid metabolism, ACP-like proteins have been detected as important factors in other reactions in prokaryotes and eukaryotes. Recently, acyl-ACP was found to be an activating factor in haemolysin A synthesis (20). Based on the analysis of amino acid sequences, the first 20 amino acids of the so called, "cytoplasmic activating factor", were identical to *E.coli* ACP. The activation of prohaemolysin A into haemolysin A, required the transfer of an acyl group from acyl-ACP to one of the internal amino acids of the prohaemolysin A. ACP was also found to be an important factor in the synthesis of membrane-derived oligosaccharides in *E.coli* (53), although, both apo and holo-ACPs appeared to be equally functional in these transglucosylation reactions (54).

ACP-like domains have been detected in polyketide synthases (PKS) (18). In some organisms polyketide synthases are multifunctional proteins (7),

whereas in others they are of a dissociable type (18). ACP domains of PKS have similar functions to ACPs in FAS, carrying acyl groups from one catalytic unit to the other. Recently, gene sequence information for the polyketide synthase from the *Saccharopolyspora erythrea* has revealed six repeated motifs whose sequences are very similar to the enzymatic domains of the PKS and FAS components (39). Each motif was found to contain coding regions for β-ketoacyl synthase, acyltransferase and ACP, whereas ketoreductase and dehydrase sequences were found only in some motifs.

Characterization of the nodulation operon region, which is confined to the symbiotic plasmid in *Rhizobium leguminosarum,* revealed that an ACP-like protein is the product of *nodF* gene (48). The gene is induced by plant root exudate and *nodD* protein. Therefore, has been postulated that the *nodF* encoded ACP could serve as a cofactor in the synthesis of cyclic glucans required for nodulation (48).

Acyl ACPs have been found as a part of the peripheral arm of the NADH:ubiquinone reductase in *Neurospora crassa* (43) and in bovine heart mitochondria (42). The ACP isolated from *N. crassa* was partially acylated with myristic and hydroxymyristic acid, which has led to speculation that mitochondrial fatty acid synthase provides some of the mitochondrial lipids (29). Independent analysis of the samples from bovine heart mitochondria under reducing and non-reducing conditions revealed that complex I contained only acylated-ACPs (42). Given that some other subunits of the mitochondrial respiratory complex have been involved in reactions other than respiration e.g. complex III in proteolytic

processing of mitochondrially imported proteins, it is possible that the peripheral arm of complex I is involved in mitochondrial fatty acid biosynthesis.

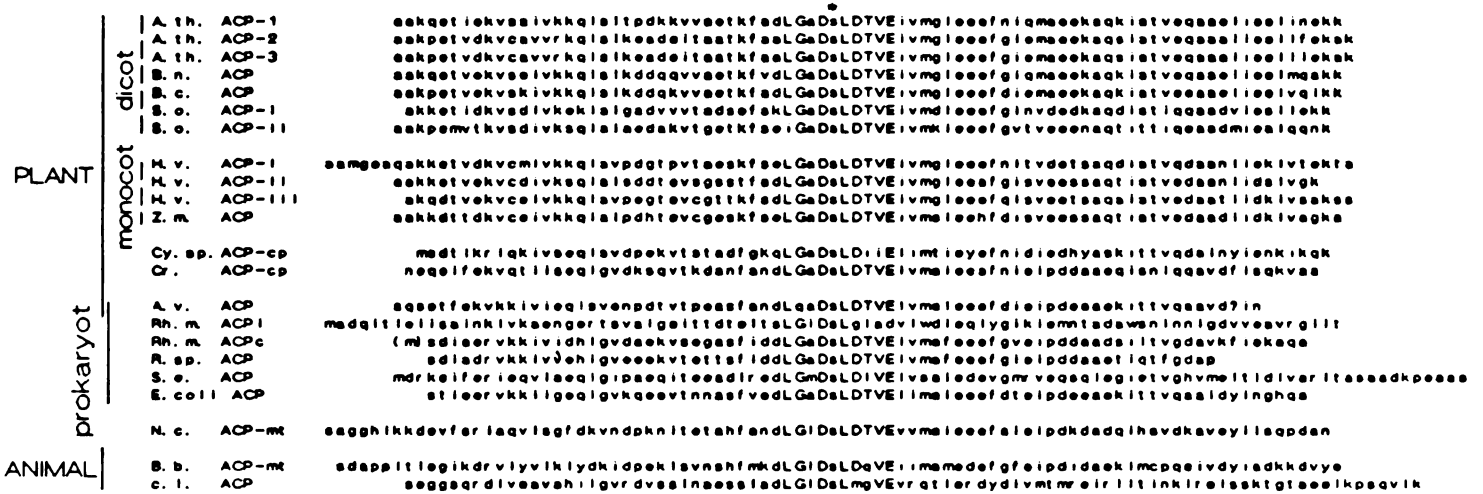
Biochemical characteristics and structure of ACPs

The first evidence for the existence of ACP in plants was obtained in 1964 by Overath and Stumpf (35). They observed that a soluble, heat stable factor similar to *E.coli* ACP was required for high rates of fatty acid synthesis in extracts of avocado mesocarp. A few years later, Simoni et al. (49) purified acyl carrier protein from spinach and avocado, determined the amino acid composition and confirmed its activity in fatty acid synthesis.

Acyl carrier proteins from plants and prokaryotes are highly similar proteins (Fig.3). They are 83 - 90 amino acid long, with molecular weights of around 10,000. All mature ACPs have a high content of acidic residues and a low content of hydrophobic and aromatic residues. As a result of their amino acid composition, ACPs are acidic proteins and therefore are highly soluble. They also bind low amounts of SDS and migrate on SDS-PAGE gels differently from their expected molecular weight. ACPs have a low isoelectric point (3.9-4.2) and are stable to heat treatment (30).

Figure 3. Comparison of the amino acid sequences of plant, animal and prokaryotic ACPs. Conserved amino acids surrounding phosphopantetheine binding site are marked with capital letters. The 4'-phosphopantetheine group is attached to a serine residue (*) near the middle of the polypeptide chain. A.th.- *Arabidopsis thaliana* (L.) Heynh. var. Columbia; B.n.- *Brassica napus*; B.c.- *Brassica campestris*; S.o.- *Spinacia oleracea*; H.v.- *Hordeum vulgare*; Z.m.- *Zea mays*; Cy. sp.- *Cylindrotheca* sp.; Cr. - *Cryptomonas*; A.v.- *Anabaena variabilis* ; Rh.m.- *Rhizobium meliloti*; R.sp.- *R. leguminosarum*; S.e. - *Saccharopolyspora erythraea*; E.coli- *Escherichia coli*; N.c.- *Neurospora crassa* ; B.t.- *Bos taurus*; c.l.- chicken liver. cp - chloroplastidial; mt - mitochondrial; monocot - monocotyledonous plants; dicot -dicotyledonous plants; prokaryot - prokaryotes.

Alignment of the mature ACP amino acid sequences



From structure/function analysis, it appears that the amino terminus is functionally more important than the carboxyl terminus (12). Deletion of more than 3 residues from the amino terminus of *E.coli* ACP resulted in a reduction of biological activity. However, deletion of more than 10 amino acid residues from the carboxyl terminus had no effect on ACP function (12). Holo ACPs have 4'-phosphopantetheine covalently bound to a serine residue through a phosphodiester bond (Fig. 2). While actively participating in fatty acid biosynthesis, acyl residues are bound to the terminal sulfhydryl of the prosthetic group via a thioester bond (30).

Predictions of protein secondary structure from the amino acid sequence data, combined with NMR studies and nuclear Overhauser data originally suggested that *E.coli* ACP has four α helices (13). However, more recent proton NMR analyses showed that the second helix is missing (23). A strong structural homology between *E.coli* and spinach ACP was recently shown by NMR studies (23) indicating that plant ACPs are more similar to prokaryotic ACPs than to the ACP regions of the animal fatty acid synthases.

Subcellular localization of acyl carrier proteins

ACP is one of the most abundant proteins in *E.coli* (30), but its subcellular localization is still not defined. In 1970, Van der Bosch et al. (55) concluded from the electron microscopic analysis of an *E.coli* auxotroph grown on [β - ^{3}H] alanine, that ACP is associated with cell

membranes. Contrary to these data, immunoelectron microscopical analysis, performed by Jackowski et al. (21), showed that ACP could be found only in the cytoplasm and did not bind to membranes. Reevaluation of the same issue combined the findings of the two groups in one. According to Bayan & Therisod (3), the majority of ACP in *E.coli* is localized in the cytoplasm. However, a small fraction (approximately 1.5% - 3% of the ACP pool) remains associated with protein binding sites in the inner membrane. The authors postulated that ACP binding sites in inner membranes are enzymes involved in phospholipid and membrane - bound oligosaccharide biosynthesis. In support of this theory, they reported that the number of ACP binding sites increases in microsomal vesicles isolated from overproducers of the glycerol-3-phosphate acyltransferase (GPAT) (4). GPAT is localized in the inner membrane and catalyzes the transfer of fatty acid chains from acyl-ACP to the *sn*-1 position of glycerol-3-phosphate in the synthesis of membrane phospholipids.

In animals and fungi, the multifunctional FAS is localized in the cytoplasm (56). Recently, ACPs have been detected as separate proteins in *N. crassa* mitochondria (43, 29) and in mitochondria of bovine heart muscle (42).

Initial reports on the localization of FAS in plants associated fatty acid synthesis with different plant organelles, due to inappropriate methods of organelle separation. In 1979, Ohlrogge et al. (34) reported that the majority of ACP is localized in plastids. However, they cautioned that because of the large proportion of the cell volume that was occupied by

plastids minor amounts of ACP present in some other organelles might not be detected. Recently, upon the localization of mitochondrial ACP in *N. crassa*, Brody and coworkers (29) were able to identify ACP-like proteins in mitochondrial fractions of pea leaves and potato tubers (6). These proteins were characterized as putative ACPs based on their cross-immunoreactivity and on the changed mobilities on PAGE upon deacylation of the protein fraction.

The highly soluble nature of ACP implied that plant ACPs are probably localized in plastid stroma. However, immunogold labeling studies using spinach ACP-I antibodies in *Brassica* leaves detected most of the ACP associated with the thylakoid membranes (50). Lack of more extensive studies on the localization not only of ACP but also of other fatty acid synthase components prevents us from making firm conclusions regarding the intracellular and intraorganellar localization of ACPs in plants.

Isoforms of acyl carrier proteins and their intercellular localization

Soon after the report that ACP from spinach leaves might be represented by more than one isoform (49), several laboratories reported that multiple ACP isoforms are present, not only in spinach (26), but also in barley (27), castor bean and soybean (24). Recent data which are based on the labeling of ACPs with [^{3}H] palmitate, detected multiple ACP isoforms even in simple multicellular plants (2).

ACP isoforms from different plants are immunologically similar (31). Antibodies raised against spinach ACP-I cross-react with spinach ACP-II and with other plant and prokaryotic ACPs. However, according to the results of a competitive binding assay, spinach ACP-II could compete with only 40% of the ACP-I binding sites (31). The same antibodies were even less efficient in recognizing ACP isoforms from prokaryotes like *E. coli*, *Anabaena variabilis* and *Synechocystis 6803* (10).

Tissue-specific expression of ACP isoforms was initially observed in spinach, soybean and castor bean (24). Based on SDS-PAGE analysis, these plants have a single ACP isoform expressed in a constitutive manner, in leaves, roots and seeds, and a single leaf-specific ACP isoform. Barley, however, has three ACP isoforms expressed in leaves (27). Their relative abundances vary greatly with the age of leaf cells (2). The predominant ACP isoform found in young regions of the leaves, becomes even more abundant as the leaf matures. The two minor ACP isoforms from the young leaf areas are less abundant as the leaf tissue gets older. These results suggest that the expression of the three ACP isoforms in barley leaves is developmentally regulated. Multiple ACP isoforms also have been detected in rapeseed (44). Cloning of ACP from a rapeseed seed cDNA library revealed the presence of a large ACP family that was mainly expressed in seeds. Low levels of the expression of the "seed-specific ACPs" were also detected in leaves (40).

In contrast to higher plants, prokaryotes and unicellular eukaryotes were, up to now, considered to have a single form of ACP. These conclusions

were based on the extensive analysis of the *E. coli* ACP (30), on the electrophoretic analysis of protein extracts from unicellular algae, *Chlamydomonas reinhardtii* and *Dunaliella tertiolecta* (2), and from photosynthetic cyanobacteria, *Synechocystis* strain 6803 (2, 22) and *Agmnellum quadruplicatum* (2), and on ACP sequence information from *Streptomyces erythraeus* (11). However, recently, two ACP isoforms were reported in *Rhizobium*. One ACP isoform, is the product of the *nodF* gene, which is part of the nodulation operon (48), and the other is a constitutively expressed ACP isoform (36). It appears that the two isoforms have different functions. The inducible ACP isoform seems to be involved in the synthesis of membrane-derived oligosaccharides, whereas the constitutive ACP isoform is part of the fatty acid synthase (48).

Molecular biology of acyl carrier proteins

Early studies characterizing ACPs suggested that plant ACPs are nuclear encoded proteins. These findings were based on differences observed in the sizes of the *in vitro* mRNA translation products compared to purified ACPs, after immunoprecipitation with antibodies to ACP (31). Isolation and characterization of other ACP clones confirmed that eukaryotic ACPs are nuclear encoded proteins. Amino acid sequence information of the purified ACP isoforms when compared to deduced amino acid sequences from cDNA and genomic clones indicated that spinach ACP-I (26, 45) and ACP-II (31, 46), barley major leaf ACP isoform (27, 16, 17) and rapeseed ACP isoform (44), are translated as precursor proteins with approximately 51-

59 amino acid long transit peptides. Furthermore, studies confirmed that the transit peptide of the precursor ACP is processed upon import into the chloroplasts. Very recently, an ACP-like sequence has been detected in chloroplast genome of the marine diatoms *Cylindrotheca* sp. Strain N1 (19) and of the *Cryptomonas* sp. (57). However, a DNA sequence search for ACP-like sequences of the tobacco chloroplast genome did not reveal any sequences similar to ACPs.

Sequence information for cDNA ACP clones from dicots [spinach ACP-I (45) and ACP-II (46), seed ACP isoforms from rapeseed (44), *B. campestris* seed (8)], and monocots [barley ACP-I (16), ACP-II (15), ACP-III (16), and maize (51)] is available. In addition to the sequence of the *E.coli* ACP gene (38), several genomic clones have been sequenced from barley (17), *B. napus* (40) and *Arabidopsis* (37, 25). The known plant ACP genes have very similar structures. The coding regions are interrupted with three introns whose positions are almost completely conserved. Intron I is located in the transit peptide coding region. Its position varies in the different ACPs by a few amino acid residues. In the barley *Acl2* and *Acl3* genes, the second intron is positioned between the last amino acid of the transit peptide and the first amino acid of the mature ACP isoform, whereas in barley *Acl1,* intron II is in between the first and second amino acid of the mature ACP-I. The ACP 09 gene from *Brassica* has a similar position of the intron as barley *Acl1*. Intron III in all known ACP genes is located only 9 base pairs downstream of the serine codon which is the binding site of the phosphopantetheine prosthetic group. The entire region surrounding the prosthetic group attachment site is highly conserved (30). The

introns from the dicot ACPs are shorter than the barley introns.

The expression patterns of spinach ACP isoforms were confirmed by Schmid and Ohlrogge (46) at the mRNA level. Sensitive RNase protection assays were used to detect ACP-II mRNAs in leaves, roots and developing seeds and ACP-I mRNA in leaves. With the isoform specific probes they were able to quantify steady-state levels of both ACP-I and ACP-II mRNA. In leaves, each of the two isoforms represented approximately 0.1 mol% of the polyadenylated RNA pool and the molar ratio between ACP-I and ACP-II messages was 0.9 (46). The measurements of steady-state levels of ACP mRNA in both spinach and soybean leaves revealed that the abundance of ACP message is higher in younger leaves than in older leaves (14). In the same study the highest relative abundance of ACP mRNA in soybean seeds was shown to be 0.027% at 20 days after flowering. In both leaves and seeds, changes in the level of ACP mRNA were accompanied by changes in the protein levels, suggesting that increases in ACP concentration in developing seeds are a result of *de novo* protein synthesis.

The transcription start sites have been determined for *B. napus* ACP 05 gene and for barley *Acl1* and *Acl3* genes. In *B. napus*, the 5' leader sequence is 69 nucleotides long. The transcription start site sequence, GGCATCA, shares five out of seven nucleotides with a proposed consensus transcriptional start sequence (40). In both barley ACP genes, *Acl1* and *Acl3*, the three transcriptional start sites have been located (17). The major transcriptional start sites in *Acl1* and *Acl3* genes result in 97 and 84 nucleotide long RNA leader sequences, respectively. In addition, minor

transcripts with 90 nucleotide and 144 nucleotide long leader sequences were detected in *Acl1*, whereas in *Acl3*, the two minor leader sequences were 77 and 140 nucleotides long. Preliminary results indicated that *Arabidopsis ACP2* message has a 123 nucleotides long leader sequence (25). No multiple transcripts were reported in the study. Putative multiple polyadenylation sites were found for barley *Acl2* and *Acl3* genes (16,17) and for spinach ACP-II (46).

The three barley ACP genes have been localized on chromosomes using the Chinese Spring wheat - Betzes barley addition lines (17, 15). According to DNA blot hybridizations, *Acl1* and *Acl2* are on a chromosome 7, approximately 10 kb apart, and *Acl3* is on the chromosome 1. All three genes were reported to be present as single copy genes. *Arabidopsis ACP2* and *ACP3* genes are linked on a 18 kb DNA fragment (25). However, no analysis has been performed to assess the number of copies for either one of them. Quantitative DNA blot hybridizations revealed a large ACP family in *B. napus* genome indicating that some genes exist in multiple copies (44). Even in spinach, which has only two to three ACP isoforms distinguishable by PAGE, DNA blot hybridizations suggest the presence of a larger gene family (46). DNA sequence comparisons indicate that *Arabidopsis ACP2* and *ACP3* genes are products of a recent gene duplication event. The degree of identity between *B. campestris, B. napus* and *Arabidopsis ACP1* genes suggests that they all share a common ancestral gene which diverged from the two other known *Arabidopsis* ACP genes.

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CHAPTER TWO

ISOLATION AND CHARACTERIZATION OF AN ACP cDNA AND A GENOMIC CLONE FROM *ARABIDOPSIS THALIANA*

Abstract

I have isolated genomic and cDNA ACP clones from λ sep6-lac5 and λ - ZAP (Stratagene) libraries, respectively. The two *Arabidopsis* ACPs, *ACP1* and *ACP2*, shared 35% identical nucleotides in the transit peptide coding region and 69% identical nucleotides in the coding region for mature ACP. By using gene specific probes derived from these two clones for the hybridization of genomic DNA blots, a minimum of three ACP genes in *Arabidopsis* has been detected. According to RNA blot analysis, *ACP1* and *ACP2* genes are expressed in leaves, roots and developing seeds. However, the possibility of the probes hybridizing to very similar, but not identical ACP RNA messages, cannot be ruled out.

Introduction

Mouse ear cress, better known as *Arabidopsis thaliana* (L.) Heynh. var. Columbia, is a small cruciferous weed, closely related to economically important crops from the genus *Brassica* (38). The small size, short

generation time and only five chromosomes of *Arabidopsis* (29,11) attracted the attention of plant geneticists who developed its extensive genetic map (21). The haploid genome of *Arabidopsis* has approximately $1x10^8$ base-pairs (1), with almost no repetitive DNA (37). Because it is the smallest known genome in higher plants, it has been postulated that *Arabidopsis* may have the minimum number of genes necessary for the growth of a plant. The initial characterizations of several *Arabidopsis* genes have supported that theory. For example, only one aldehyde dehydrogenase gene was identified in the *Arabidopsis* genome, whereas two or three have been found in other higher plants (7). Chlorophyll a/b light-harvesting protein, that has a large gene family with eight and a minimum of sixteen genes in *Pisum* and *Petunia*, respectively, has only three genes in *Arabidopsis* (26). Similarly, the gene family for the 12S-globulin seed-storage protein genes has a lower number of genes in *Arabidopsis* than in other plants (29). However, recently several large gene families in *Arabidopsis* have been characterized that were of comparable size to the gene families from other plants. Both α and β-tubulin genes in *Arabidopsis* are organized in large gene families that have a minimum of six and nine genes, respectively (22,46). Even though it appears that more exceptions to the general rule of smaller gene families in *Arabidopsis* will be found, it is still likely that most *Arabidopsis* gene families have a reduced number of genes.

For many years *Arabidopsis* has been a model plant for the analysis of lipid metabolism. A large number of mutants in various steps of lipid biosynthesis provided valuable information on the biosynthesis of lipids (32). In *Arabidopsis*, as in other higher plants, the membrane lipids are

synthesized by two complementary pathways that are present in plastidial and cytoplasmic compartments (40,6). The origin of the synthesis of particular glycerolipids such as phosphatidylglycerol, monogalactosyldiacylglycerol, digalactosyldiacylglycerol and sulfolipids, could be revealed from the positional distribution of 16 carbon and 18 carbon fatty acids (48). The glycerolipids that are made predominantly in plastids contain substantial amounts of hexadecatrienoic fatty acid at the *sn*-2 position. On the contrary, the glycerolipids made in the endoplasmatic reticulum contain predominantly α-linolenate at the same position. As a result of those differences the group of plants whose plastid membrane lipids are assembled in the extraplastidial compartment are named "18:3 plants", as opposed to the "16:3" plants, where plastids contribute to the synthesis of glycerolipids. In *Arabidopsis*, a "16:3" plant according to Browse et al. (6), 38% of *de novo* synthesized fatty acids goes into the plastidial pathway and more than half of the fatty acid pool (56%) that is exported to the cytoplasm re-enters the chloroplasts where it contributes to the production of thylakoid glycerolipids. *Arabidopsis* leaf lipids contain predominantly polyunsaturated 18 carbon fatty acids, whereas its seeds have in addition to the 18 carbon polyunsaturates, 20 and 22 carbon fatty acids (5).

In spite of the extensive work on lipid metabolism in *Arabidopsis*, very little information is available on the regulation of fatty acid biosynthesis. Having in mind the advantages of *Arabidopsis* for molecular biological and biochemical studies, I wanted to characterize *Arabidopsis* ACP genes as a first step that will bring us closer to the understanding

of the regulation of the expression of the components of fatty acid synthase. Therefore, I began to characterize acyl carrier proteins in *Arabidopsis*, so they can be used as markers in the analysis of fatty acid biosynthesis in plants (34). It has been known that *Arabidopsis*, like other multicellular plants, has multiple ACP isoforms that appear to be expressed in a tissue-specific manner (2). However, the specific functions of ACP isoforms have not yet been revealed even in spinach where the characterization of ACPs led to the amino acid sequencing of the ACP-I (24) and ACP-II (33) isoforms and to the cloning of their cDNA clones (43,44).

Experimental procedures

Materials. An *Arabidopsis* genomic library in λ sep6-lac5 was provided by Chris Somerville. An *Arabidopsis* expression library in λ-ZAP (Stratagene) prepared from mRNA isolated from cold-acclimated plants was a gift from Mike Tomashow. A cDNA ACP clone from *Brassica napus* seeds, pBN45, was donated by Richard Safford. A 66-nucleotide long oligomer, complementary to the region encoding the 22 amino acids surrounding the phosphopantetheine binding site (15), was a gift from Penny von Wettstein-Knowles and Lars Hansen.

Plant material. *Arabidopsis thaliana* (L.) Heynh. ecotype Columbia plants were grown under a 16 h light/dark cycle either in a soil or in hydroponic culture. Plants grown in the soil were watered with a Hoagland solution

(5 mM KNO_3, 2.5 mM KPO_4, pH 5.5, 2 mM $MgSO_4$, 2 mM $Ca(NO_3)_2$, 2.5 mM Fe.EDTA, 70 μM H_3BO_3, 14 μm $MnCl_2$, 0.5 μM $CuSO_4$, 1 μM $ZnSO_4$, 0.2 μM $NaMoO_4$, 10 mM NaCl, 0.01 μM $CoCl_2$) once a week. Hydroponic culture was set in a Hoagland solution with a continuous aeration and weekly changing of the solution. Seeds of the hydroponically grown plants were germinated on a sheet of a cheese cloth that was 0.5 cm above the water layer.

Preparation of hybridization probes. DNA fragments that were used for random primer labeling were resolved by agarose gel electrophoresis using Sea Plaque low-gelling agarose (FMC Bioproducts) in Tris-Acetate buffer. The band of interest was cut from the gel, melted at 70^0C, and used directly for labeling. Random primer labeling reactions were carried out with a mix of random hexamers, Klenow fragment and (^{32}P)dCTP, according to the manufacturers' protocols (US Biochemical Corp.). Oligonucleotides were either random primer labeled with [^{32}P]CTP or end-labeled with [^{32}P]ATP using Klenow fragment according to the methods described in Maniatis (42).

Sense and antisense RNA probes were made according to the protocols included with a Promega Riboprobe kit, utilizing either T3, T7 or Sp6 RNA polymerase. For templates, recombinant plasmids, were CsCl purified and linearized with one of the restriction enzymes from the polylinker region (42). The enzymes were inactivated by proteinase K treatment and removed by phenol/ chloroform extraction. The DNA fragments were recovered by ethanol precipitation. Incorporation of the (^{32}P)UTP into RNA probes was determined by Cerenkov counting of aliquots spotted on glass fiber filters

and precipitated with 5% TCA containing 20 mM sodium pyrophosphate (4). The length of the probes was confirmed by resolving labeled RNA probes in the 6 M urea PAGE followed by autoradiography.

Screening of the *Arabidopsis* genomic library. $1x10^6$ plaque forming units of the λ library were adsorbed to $5x10^6$ *E.coli* LE392 in SM buffer for 20 minutes at 37^0C. 3ml of 0.7% top agarose, that was cooled to 55^0C, was mixed with cells and the mixture was spread over the LB plates. Plates were then incubated at 37^0C for 14-18 hours. When the clear plaques became visible, plates were either stored at 4^0C or processed immediately. The nitrocellulose circles were marked and placed face-down onto a plate for 1 minute. Replica filters were left on the top agar layer for 90 seconds. Each filter was carefully removed and placed face-up for 1 minute on a sheet of filter paper saturated with 0.5 M NaOH, 1.5 M NaCl for DNA denaturation. Nitrocellulose filters were then neutralized with an incubation on 1.5 M NaCl, 0.5 M Tris HCl, pH 8.0 for 5 minutes. Following the neutralization step, filters were rinsed in 2x SSPE and baked for 2 hours at 80^0C in a vacuum oven.

Nitrocellulose filters were prehybridized at 42^0C in 7% SDS, 2x SSPE, 30% formamide for 30 minutes to 5 hours. The prehybridization mixture was removed and a the same buffer and [^{32}P]UTP labeled RNA probe derived from pBN45 plasmid ($2x10^6$ CPM) were added to the container. Hybridization was performed at 42^0C for 12-24 hours. Filters were washed twice at room temperature with 2x SSPE, 0.2% SDS and once at 50^0C for 30 minutes in 1x SSPE, 0.2% SDS. Filters were exposed to film for 2 days with intensifying

screens at -70^{0}C.

Antibody screening of the *Arabidopsis* cDNA expression library. The cDNA library made in λ-ZAP was grown in *E. coli* strain BB4 (Stratagene). Cells that were prepared for the adsorption of viruses were grown in TBMg + maltose media and harvested by centrifugation at 3000 rpm for 10 minutes. The pellet was resuspended in 5 ml 10 mM $MgSO_4$. Viral dilutions that were made in SM buffer were mixed with *E.coli* cells and incubated with shaking at 37^{0}C for 20 minutes. 0.7% molten agarose, cooled to 55^{0}C, was mixed with cells and the mixture was poured on the agar plate. Plaques were grown at 42^{0}C for 6-8 hours. Dry nitrocellulose filters that were saturated with 10 mM IPTG solution, were placed onto the plates. IPTG induced the expression of the proteins during the incubation of the plates at 37^{0}C for 2-4 hours. At the end of incubation, filters were transferred to petri dishes. Proteins were fixed on the filters with 3% TCA for 5 minutes. Residual amounts of TCA were removed by rinsing filters with dH_2O three times.

Before immunoblotting with spinach ACP-I or recombinant spinach ACP-I antibodies, filters were blocked for 2 hours in Blotto. 1:250 dilution of the primary antibody was prepared in Blotto, and filters were immunoblotted for one hour. At the end of incubation with primary antibodies, filters were washed 3 times in TBST for 10 minutes each. Secondary goat anti-rabbit IgG antibodies were diluted to 1:1000 and added to the filters. After one hour of incubation, the antibody solution was removed and filters were washed three times for 10 minutes with TBST and

once with alkaline phosphatase buffer (100 mM Tris Hcl, pH9.5, 100 mM NaCl, 5 mM $MgCl_2$). Color development was done in alkaline phosphatase buffer with NBT (50 mg/ml) and BCIP (50 mg/ml) that were dissolved in 70% and 100% N,N-dimethylformamide, respectively. The color development was stopped by thoroughly rinsing filters with dH_20. Positive plaques were picked from the plates, and phage was extracted from the cells in SM buffer supplemented with a few drops of chloroform.

Putative positive plaques were rescreened with anti-spinach ACP-I antibodies, and replica filters were probed with [^{32}P]ATP end-labeled 66-nucleotide long oligomer. Phagemids from the plaques that gave positive signal with antibodies and with the 66-mer were isolated and pBluescript plasmids were rescued using R408 helper phage according to the Stratagene protocol. Positive plasmids were confirmed by probing with random primed (^{32}P)dCTP-labeled 66-mer.

Preparation of λ DNA. The putative positive plaques were grown in 2 ml LB liquid cultures. 1/20 volume of the phage stock, that was prepared in 1 ml of SM, was mixed with 500 μl SM and with $1x10^8$ bacteria, and incubated at 37^0 for 20 minutes. 2 ml of LB media, that was supplemented with $MgSO_4$ to 5mM, was added to the mixture of bacteria and phage, and left to grow overnight at 37^0C at constant shaking. After 10 hours of growth, approximately four drops of chloroform were added to the cells, which were left in the shaker for an additional 15 minutes. Following centrifugation at 3,000 rpm for 5 minutes, the supernatant was transferred to another tube where it was treated with DNase and RNase for 20 minutes at 37^0C.

RNase was inhibited with the addition of 40 μl DEPC. Following the addition of 75 μl of 10% SDS and 375 μl of 2 M Tris HCl, 0.2 M EDTA, the solution was mixed and placed on ice for 30 seconds. 250 μl of 8 M potassium acetate was added to the solution which was then mixed by inversion, and the solution was put on ice for an additional 30 seconds. Proteins were pelleted by centrifugation at 10,000 rpm for 15 minutes. DNA from the supernatant was precipitated with 2 volumes of ethanol and spun down at 10,000 rpm for 10 minutes. The DNA pellet was then washed with 70% ethanol and resuspended in TE buffer.

DNA subcloning and sequencing. DNA of the putative λ ACP clone was digested with *Eco*RI and subcloned into pBS(+) (42). A restriction map of the 4.8 kb *Eco*RI fragment, which hybridized to the [^{32}P]RNA probe derived from pBN45, was constructed using single, double and triple digests with several restriction enzymes. DNA fragments were resolved by 1% agarose gel electrophoresis and DNA blots were hybridized with [^{32}P]-labeled 66-mer.

A putative ACP clone that was isolated from the λ ZAP expression library was digested with *Eco*RI. The *Eco*RI fragment that hybridized to the [^{32}P]-labeled 66-mer, was subcloned into pBS(+)KS for further restriction mapping and sequencing.

DNAs of plasmid clones that were used for sequencing were purified either by CsCl gradient purification or by a "mini" preparation method that was

followed by PEG precipitation of DNA (42). DNA was alkaline denatured and sequenced following the dideoxy chain termination method using either G mix or I mix and Sequenase 2.0 according to the U.S. Biochemical protocols.

Preparation of *Arabidopsis* genomic DNA. Either fresh or frozen aerial parts of 5 week old *Arabidopsis* plants were used for the isolation of genomic DNA. 10 g of tissue were ground with mortar and pestle in liquid nitrogen. The frozen powder was added to 10 ml of homogenization buffer (0.15 M Tris HCl, pH 8.5, 0.1 M EDTA, 2% N-lauroylsarcosine, sodium salt, 0.1 mg/ml proteinase K). The mixture was incubated at 37^0C for 30 minutes with gentle stirring. Cell debris was removed by centrifugation at 10,000 rpm for 10 minutes. The supernatant was transferred to another tube, while the pellet was again resuspended in homogenization buffer and incubated at 37^0C for 30 minutes. After a second centrifugation the supernatants were combined, and the DNA was precipitated with 2 volumes of 100% ethanol at 4^0C for 2-3 hours. DNA was pelleted by centrifugation at 5,000 rpm for 60 minutes. The pellet was resuspended in 10 mM Tris HCl, pH 8.0, 1 mM EDTA and DNA was purified on a CsCl gradient containing ethidium bromide, for 18 hours at 45,000 rpm. Upon completion of the centrifugation, the ethidium bromide was extracted from the gradient with water-saturated 1-butanol. DNA was precipitated with 70% ethanol at 4^0C for 3-4 hours and after collection by centrifugation the pellet was resuspended in TE buffer. Quantification of DNA was performed by measuring absorbance at 260 nm and 280 nm.

Preparation of *Arabidopsis* RNA. Five to six week old plants were used for the extraction of total RNA from leaves. Developing seeds were collected from the seed pods with pale green to green seeds from the 5- to 8- week-old *Arabidopsis* plants. Two-week-old hydroponic cultures of *Arabidopsis* were used as a source of roots for RNA isolation. All tissues were flash frozen in liquid nitrogen and stored at -70^{0}C until used.

Total RNA was extracted from 400 mg of root material and 200 mg of leaf or developing seed as described in Hall *et* al. (16) with some modifications. Tissue was ground in a microfuge tube with a minipestle in a homogenization buffer (0.2 M Tris HCl, pH 9.0, 0.4 mM NaCl, 25 mM EGTA, 1% (w/v) SDS) which was preheated in boiling water. Sequentially, 5 mg/ml of PVP (mol wt 360,000) and 0.5 mg/ml proteinase K were added to the homogenate. After incubation at 37^{0}C for 1 hour, proteinase K was inhibited by the addition of 12 mg/ml $BaCl_2$ and incubation on ice for 15 minutes. At the end of incubation, KCl was added to a final concentration of 143 mM, and the homogenate was centrifuged for 10 minutes at 15,000 rpm. RNA from the supernatant was precipitated overnight in 2 M LiCl. After centrifugation for 20 minutes at 15,000 rpm, the pellet was rinsed with 2 M LiCl and resuspended in DEPC-treated water. Total RNA was stored in ethanol at -70^{0}C. Before use, the RNA was dissolved in DEPC-treated water.

DNA and RNA gel electrophoresis and blotting techniques. Purified plasmid, phage or genomic DNAs were digested with one or more restriction endonucleases, according to the conditions recommended by the

manufacturer, and were resolved in a 0.8 % or 1% agarose gel in TE buffer under constant current (60 - 120 mA). The upper region of the gel containing the high molecular weight DNA fragments was exposed to UV for 5 minutes to nick the DNA and enhance transfer of the large size DNA fragments (42). Prior to DNA transfer to a nitrocellulose filter, DNA was denatured by soaking the gel in 1.5 M NaCl, 0.5 M NaOH for 30 minutes. The gel was neutralized by a 30 minute incubation in 0.5 M Tris HCl, pH 7.5, 1.5 M NaCl. The transfer of DNA was done in 10x SSPE. The filter was baked for an hour at 80^0C in a vacuum. In some cases DNA was transferred from the gels to the nitrocellulose by alkaline transfer. Gels were rinsed for 15 minutes in dH_2O, and the transfer and simultaneous denaturation of DNA was done in 0.4 M NaOH. Upon completion of the transfer, the membrane was briefly washed in 2x SSPE and air dried.

Total RNA was subjected to denaturing formaldehyde/formamide 1% agarose gel electrophoresis (42). Following electrophoresis, the gel was washed in DEPC-treated water for 15 minutes. The transfer of RNA to the Zeta probe (Bio Rad) nylon membrane was done in 5 mM NaOH. Membranes were first air dried and then vacuum dried at 80^0C for 1 hour.

Prehybridization and hybridization of the DNA blots were done at 50^0C for 16 and 24 hours, respectively, in 2xSSPE, 7% (w/v) SDS, 30% (v/v) formamide. [^{32}P]UTP-labeled RNA probes ($2x10^6$ CPM) were added to the hybridization mix. Following hybridization, the DNA blot was washed twice at room temperature for 5 minutes in 2xSSPE, 0.2 % (w/v) SDS, and once for 30 minutes at 50^0C in 1x SSPE, 0.2% (w/v) SDS. Blots were exposed to films

for variable time periods at -70^{0}C with intensifying screens.

RNA blots were prehybridized in 5xSSPE, 1% (w/v) SDS, 15x Denhardt's, 50% (v/v) formamide and 0.5 mg/ml salmon sperm DNA at 65^{0}C for 6 to 16 hours and then hybridized with a [^{32}P]UTP-labeled antisense RNA probe in the fresh prehybridization buffer for 24 hours at 65^{0}C. The filter was washed with two five minute washes at room temperature in 2x SSPE, 0.1 % (w/v) SDS, and with one, 30 minute wash at 65^{0}C in 0.2x SSPE, 0.1% (w/v) SDS.

RESULTS

Structure of the *ACP1* gene. Approximately 1x10^5 plaques of the *Arabidopsis* genomic library were screened with a [^{32}P]RNA probe prepared from the *B. napus* ACP cDNA clone, pBN45, as a heterologous probe (41). DNA was isolated from three positive clones and was digested with *Eco*RI. Fragments were resolved on an agarose gel, transblotted to nitrocellulose and hybridized against the same probe used for library screening. *Eco*RI fragments that hybridized to the probe were subcloned into the pBS(+) vector (Fig. 4). The subclones, pAD4 and pλ-5, were further analyzed. A partial restriction map of the 2 kb *Eco*RI fragment of pλ-5, followed by DNA blot hybridizations, identified a region of the clone that hybridized to the probe. However, after sequencing 95% of this region there was no significant identity with any known ACP sequences. Therefore, it was concluded that the clone pλ-5 was a false positive.

Single, double and triple digests with 6 restriction endonucleases of the 4.8 kb *Eco*RI fragment from the pAD4 were resolved on agarose gels. Gels were transblotted to nitrocellulose and the DNA was hybridized to [^{32}P]-labeled pBN45 derived RNA probe. Information from the agarose gels and DNA blot hybridizations was used for the construction of a restriction map and for the generation of subcloning strategy (Fig. 4). Double stranded DNA sequencing of approximately 30% of the region of the 4.8 kb *Eco*RI fragment revealed a full length ACP gene (Fig. 5), termed *ACP1* (36, see Appendix).

Based on DNA sequence comparisons with previously published ACP cDNA sequences from *B. napus* (41) and from *B. campestris* (39), it was deduced that the *ACP1* gene has three AT-rich introns. One intron is positioned in the transit peptide coding region, and the two other introns are in the coding region for mature ACP (Fig. 5). Intron-I is located between codons for amino acids 16 and 17. Intron-II separates the first and the second amino acid of the mature polypeptide, whereas intron-III is located within the conserved prosthetic group attachment region. The longest intron is intron-I: 445 bp. Introns II and III are similar in size, 80 and 76 bp, respectively. Splice site sequences are similar to the consensus sequences for the intron splice sites from dicots (13). The sequence surrounding the most likely translational start codon correlates well with both Kozak's (23) and Lutcke's (27) consensus sequences for the translational start site from animal and plant genes, respectively. At 106 bp upstream from the translational start site is a putative TATA box (23). A sequence that resembles a polyadenylation site consensus sequence

Figure 4. Restriction map of pAD4 and sequencing strategy. RI - *Eco*RI, N - *Nco*I; H - *Hin*dIII; B - *Bgl*I; S - *Sac*I; Sl - *Sal*I. Exons are marked with closed boxes and introns are marked with open boxes. ATG - putative translational start codon; TAA - putative translational stop codon; Ser - prosthetic group binding site.

Restriction map and sequencing strategy of pAD4

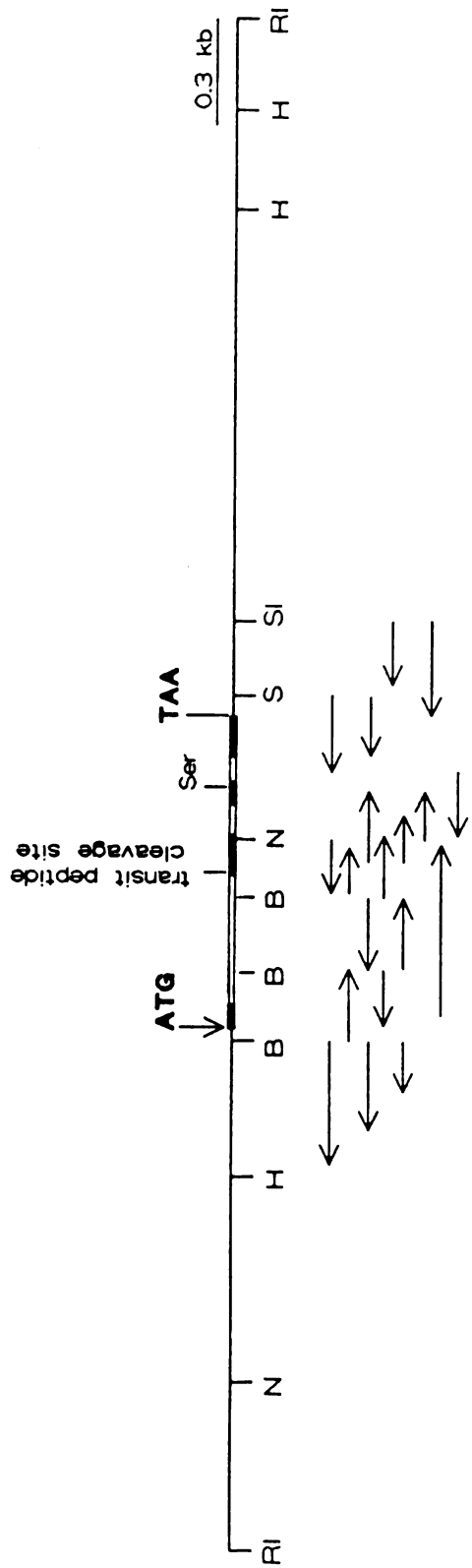
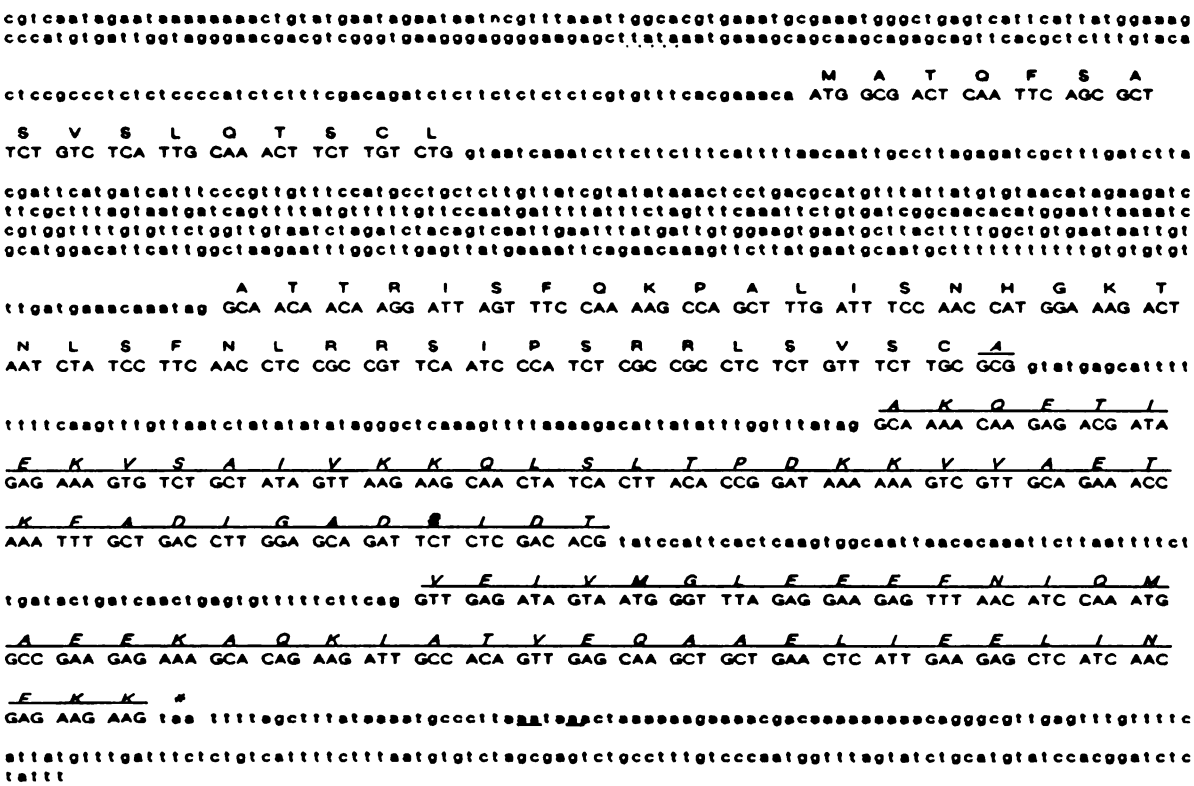


Figure 5. DNA and deduced amino acid sequence of *Arabidopsis ACP1* gene. The coding region of the pre ACP-1 is marked with capital letters. The coding region for mature ACP-1 is underlined with a solid line. Putative TATA box is underlined with a dotted line. Prosthetic group attachement site is marked with a bold letter (S). Putative polyadenylation consensus sequence is underlined with a dashed line.

DNA and deduced amino acid sequence of *Arabidopsis ACP-1* gene



(AATAAA) (18) is only 25 bp downstream from the stop codon.

Structure of the *ACP2* cDNA clone. $6x10^4$ plaques from the *Arabidopsis* cDNA expression library made in λ - ZAP were screened using two rabbit antisera prepared against spinach ACP-I (25) and recombinant spinach ACP-I (3). Twelve putative positive clones were further screened with anti-spinach ACP-I antibodies and with [^{32}P]ATP end-labeled 66-mer. Two recombinant phagemids that were picked as the putative ACP clones were excised according to the Stratagene protocol. DNA was digested with *Eco*RI and analyzed on an agarose gel. Only one of the two clones hybridized to the probe. Partial restriction mapping revealed that the clone, pAL120-7, has two *Eco*RI fragments. Based on the DNA blot hybridization data the 0.7 kb *Eco*RI fragment, which hybridized to the end-labeled 66-mer, was subcloned. A partial restriction map of the newly formed pAL120-E (Fig. 6) was used to develop a strategy for sequencing. DNA sequencing of the *Eco*RI fragment of the pAL120-E clone revealed a 716 bp fragment with an open reading frame encoding 136 amino acids (Fig. 7).

Both the nucleic acid and deduced amino acid sequences have 70% identity with *Arabidopsis* ACP-1, indicating that these two clones encode substantially different ACPs (Table 1). 16 out of 19 amino acids from the highly conserved region surrounding the prosthetic group binding site of ACP-2 were identical to the amino acid composition of the same region from other plant ACPs (Fig. 3). ACP-2 has a 52 amino acid transit peptide based on the sequence comparisons between the putative translational start site and N-terminal amino acid sequences of other mature ACPs. Recently,

Figure 6. Restriction map and sequencing strategy for pAL120-E. RI - *Eco*RI; H - *Hin*dIII; S - *Sac*I. ATG - the putative translational start codon; TAG - the putative translational stop codon; Ser - prosthetic group binding site.

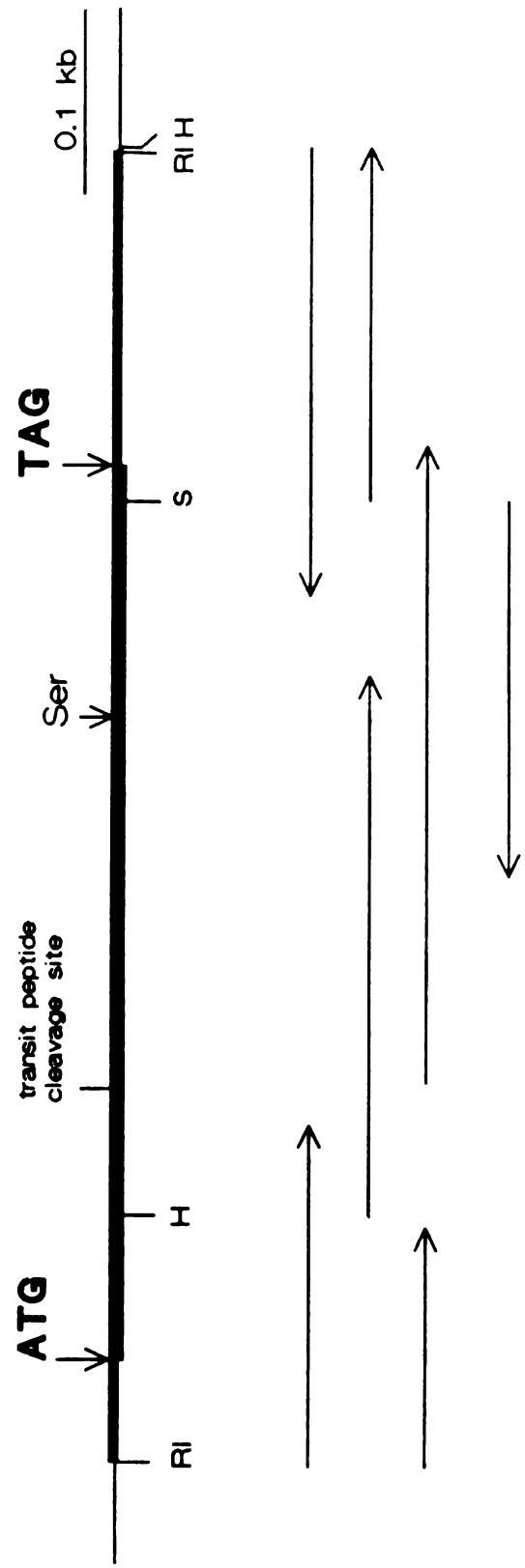

0.1 kb
RI H
TAG
S
Ser
transit peptide
cleavage site
H
ATG
RI

Figure 7. DNA and deduced amino acid sequence of an *Arabidopsis ACP2* cDNA clone, pAL120-E. The mature ACP-2 coding region is underlined with a solid line. The putative prosthetic group binding site is marked with a bold S (S). The putative termination codon is marked with an asterix. The putative polyadenylation consensus sequence is marked with a dashed line.

DNA and deduced amino acid sequence of *Arabidopsis ACP-2* cDNA clone

```
                                                                                ctcttcgtcttctccgcctccttcaaatctcactccgatctctctacgattcattcgttct

 M   A   S   I   A   A   S   A   S   I   S   L   Q   A   R   P   R   Q   L   I   A
atg gct tcc att gct gct tct gct tct att tcc ctt caa gct cgt cct cgc caa ctg gca gcg

 A   S   Q   V   K   S   F   S   N   G   R   R   S   S   L   S   F   N   L   R   Q
gct agt caa gtt aaa agc ttt agc aat gga aga aga agc agt ctt gac ttg ggt ctc cgc cag

 L   P   T   R   L   T   V   S   C   A   A   K   P   E   T   V   D   K   V   C   A
ctt cct acc cgc ttg act gtt tcc tgc gct gca aaa cct gag aca gtg gac aag gtg tgt gca

 V   V   R   K   Q   L   S   L   K   E   A   D   E   I   T   A   A   T   K   F   A
gtt gtc aga aag caa ctc tca ctc aaa gag gct gac gaa att acc gct gcc acc aaa ttt gct

 A   L   G   A   D   *   L   D   T   V   E   I   V   M   G   L   E   E   E   F   G
gca ctt ggt gct gat tcc ctt gat acg gtg gag ata gtt atg gga tta gag gaa gag ttt ggg

 I   E   M   A   E   E   K   A   Q   S   I   A   T   V   E   Q   A   A   A   L   I
att gaa atg gcg gag gag aaa gca cag tct atc gcc aca gtt gag caa gca gct gca ctc att

 E   E   L   L   F   E   K   A   K   *
gag gag ctc ttg ttg gaa aag gcc aag tag aatatctttattacattagcgaaaaaggcaaaaaaatcaaaaccc

aaaccactatcttattgtttcgttagctagagaycaattgtgtctgttgaaagatttttatgttatttttggggaaattattacaa
ggcttgtatctgacttttatctttcttctaaaacatttttactttcagccttcatcaaaactctcttacgaaacttatgtagtctc
atttcctaagtaaagatgtttttccctttgaag
```

the sequence of an *Arabidopsis* genomic ACP clone, A1, analogous to *ACP2* has been reported (25). However, the genomic ACP clone did not have any apparent polyadenylation consensus sequences. The ACP-2 cDNA clone, pAL120-E, has 78 base pairs of additional sequence information at the 3' nontranslated end, and a putative poly(A+) consensus site (18) is located 220 nucleotides away from the stop codon.

All known Brassicaceous ACPs have an identical sequence of seven amino acids (met-ala-glu-glu-lys-ala-gln) located seventeen amino acids downstream from the amino acid serine to which the prosthetic group binds (Fig. 3). In addition, both *Arabidopsis* ACPs have higher sequence similarity at the carboxy terminal regions (79% over the last 28 amino acids) than do the amino terminal regions (54% over the first 28 amino acids of the mature protein). Similar levels of sequence conservation have been found in other plant ACPs.

DNA blot hybridizations. *Arabidopsis* genomic DNA was digested to completion with *Eco*RI and *Hin*dIII. DNA fragments were resolved by 1% agarose gel electrophoresis and blotted to nitrocellulose membranes. Identical DNA blots were probed with [^{32}P]UTP labeled RNA probes synthesized *in vitro* from either a full length *Arabidopsis* cDNA ACP clone, pAL120-E, or from a *Sal*I deletion, pAD430-A, of the *ACP1* genomic *Arabidopsis* clone, pAD4. The pAD430-A plasmid contained the complete *ACP1* coding region with introns. Under the hybridization conditions used these two clones do not cross-hybridize.

Table 1. DNA percentage identity of the transit peptide and mature ACP coding regions. *Arabidopsis* AD4 ACP - genomic *Arabidopsis ACP1* clone (36); *Arabidopsis* AL 120-E ACP - cDNA *Arabidopsis ACP2* (25); *Arabidopsis* A2 ACP - genomic *Arabidopsis ACP3* clone (25); *B. napus* ACP05 - genomic ACP clone (9); *B. campestris* cDNA ACP - seed cDNA ACP clone (39); *S. oleracea* ACP-I - leaf cDNA ACP clone (43); *S. oleracea* ACP-II - root cDNA ACP clone (44); *H. vulgare* ACP-I - leaf cDNA ACP clone (15).

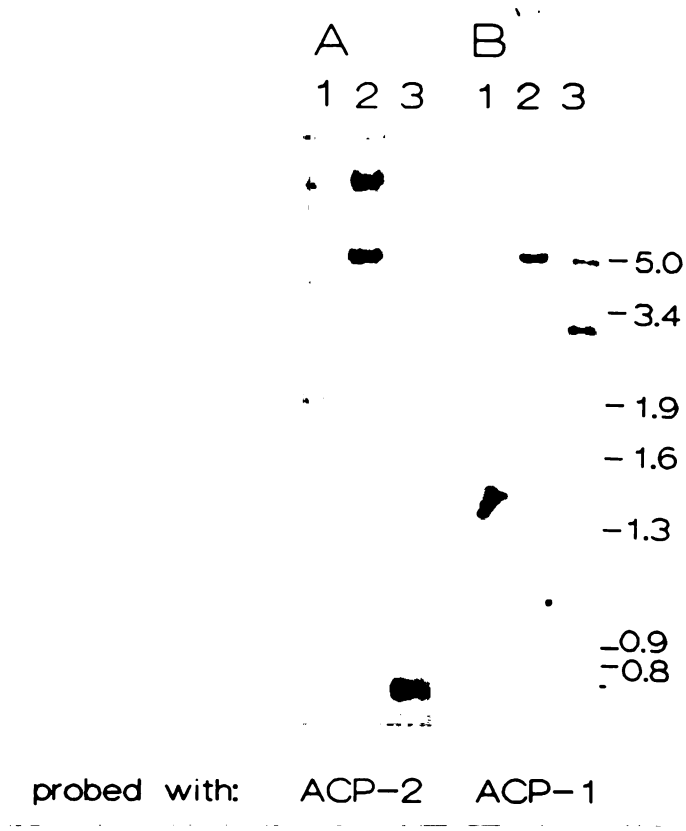
transit pept. / mature pept.	*A. thaliana* ACP-1	*A. thaliana* ACP-2	*A. thaliana* ACP-3	*B. napus* ACP05	*B. campestris* cDNA ACP	*S. oleracea* ACP-I	*S. oleracea* ACP-II	*H. vulgare* ACP-I	*H. vulgare* ACP-III
A. thaliana ACP-1		35	35	57	42	36	38	37	40
A. thaliana ACP-2	**69**		90	40	40	35	37	37	37
A. thaliana ACP-3	**69**	**96**		36	37	36	37	35	38
B. napus ACP05	**83**	**71**	**72**		73	37	37	35	39
B. campestris cDNA ACP	**82**	**69**	**70**	**87**		37	39	37	37
S. oleracea ACP-I	**57**	**60**	**60**	**63**	**62**		35	39	36
S. oleracea ACP-II	**58**	**62**	**61**	**63**	**62**	**64**		36	40
H. vulgare ACP-I	**60**	**61**	**60**	**65**	**65**	**65**	**61**		40
H. vulgare ACP-III	**59**	**67**	**64**	**63**	**62**	**61**	**63**	**67**	

The *ACP1* probe hybridized to a single fragment in both *Eco*RI and *Hin*dIII digested genomic DNA (Fig. 8b). The 4.8 kb *Eco*RI and the 3.2 kb *Hin*dIII fragments hybridizing to the probe were identical in size to the *Eco*RI and *Hin*dIII fragments identified by restriction mapping, respectively. These data indicated that the *Arabidopsis* genome has a single-copy gene homologous to the pAD4 clone.

When *Arabidopsis* genomic DNA was digested with *Hin*dIII or *Eco*RI, respectively, there were three or two fragments detected on DNA blots probed with *ACP2* clone (Fig. 8a). The sequence information of the two *Arabidopsis* ACP genomic clones that were recently reported (25) helped to unravel the information provided by the DNA blot hybridizations. One of the two *Arabidopsis* ACP genomic clones isolated by Lamppa and Jacks (25), A1, is the genomic equivalent of the *ACP2* cDNA clone, pAL120-E, described here. In addition, sequence information confirms that A1 is linked to an *ACP3* gene (named A2 by Lamppa et al.). The linked genes, which have more than 90% sequence identity, both have a *Hin*dIII enzyme recognition site 60 nucleotides downstream of the translational start site in the transit peptide coding region. In contrast, only the *ACP3* gene has an *Eco*RI site in its 5'untranslated region. Therefore, the three fragments detected in the *Hin*dIII digest and two fragments from the *Eco*RI digest represent *ACP2* and *ACP3* genes.

Genomic DNA blots were also probed with random primer labeled 66-mer. At lower stringency conditions, the probe hybridized to a large number of bands. Most of the hybrids melted at increasing temperatures. Even

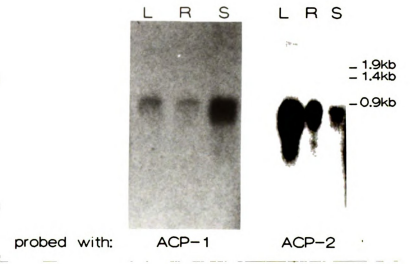
Figure 8. DNA blot hybridization analysis of ACP genes in *Arabidopsis*. *Arabidopsis* genomic DNA was digested either with *Hin*dIII (lane 1) or *Eco*RI (lane 2). *Eco*RI digests of pAL120-E and pAD430A were used as controls. Identical blots were probed either with [^{32}P]-labeled RNA probe derived from *ACP2* cDNA clone, pAL120-E (blot A) or *ACP1* genomic clone, pAD430A (blot B). Positions of the λ *Hin*dIII/*Eco*RI size markers are labeled on the right hand side.


A
B
1 2 3 1 2 3
5.0
3.4
1.9
1.6
1.3
0.9
0.8
probed with: ACP-2 ACP-1

though the 66-mer was found to be a relatively universal ACP probe for the cDNA clones, in hybridizations against genomic sequences it is a weak probe. The oligonucleotide is designed to complement the highly conserved region that surrounds the phosphopantetheine binding site of the ACP. However, in seven sequenced plant genomic ACP clones (two from barley (15), three from *Arabidopsis* (25,36), and two from *B. napus* (9)), this region is interrupted by an intron. Even though it is not known whether this occurs in every ACP gene, the poor hybridization to DNA blots with labeled 66-mer strongly suggests that it may be.

RNA blot hybridizations. Both immunoblot and RNA blot analysis of ACP expression in spinach revealed the presence of tissue-specific and constitutive ACP isoforms (44,43). Similarly, immunoblot results of proteins separated on SDS PAGE suggested that *Arabidopsis* might also have tissue-specific and constitutive ACP isoforms (2). In order to address this question, total RNAs isolated from leaf, root and developing seed material were size-fractionated on denaturing formaldehyde/formamide 1% agarose gels. The RNAs were blotted to nitrocellulose and the filters were hybridized with [^{32}P]UTP-labeled antisense RNA probes made either from pAL120-E or from pAD430-A. In both cases, RNA probes hybridized to the same length mRNA in RNA extracts from all three tissues (Fig.9). However, because of the high degree of identity between ACP genes, detected mRNA may result either from a single gene expressed constitutively in all three tissues or from very similar ACP genes (e.g. *ACP2* and *ACP3*) expressed in a tissue-specific manner.

Figure 9. RNA blot hybridizations of the total RNA purified from *Arabidopsis* leaves, roots and developing seeds. Total RNA was size-fractionated on formaldehyde/formamide agarose gels. RNA blots were probed with either [^{32}P]-labeled antisense RNA probes derived from *ACP2* cDNA clone, pAL120-E (blot A) or *ACP1* genomic clone, pAD430A (blot B). Lanes 1 to 3 contain total RNA from leaves (L), roots (R) and developing seeds (S), respectively.


L R S
L R S
1.9kb
1.4kb
0.9kb
probed with:
ACP-1
ACP-2

DISCUSSION

Two ACP clones were isolated by screening the *Arabidopsis* genomic and cDNA libraries, respectively. The 70% nucleic acid and deduced amino acid sequence identities found between these two clones indicated that they encode different ACP isoforms. Recently, Lamppa and Jacks (25) have characterized two *Arabidopsis* ACP genomic clones, A1 and A2. The A1 clone is a genomic analog of the *ACP2* cDNA clone, pAL120-E, and thus in further text, A1 and A2 are referred to as *ACP2* and *ACP3* (see Appendix). *ACP2* and *ACP3* genes are linked and have only 10% difference in their nucleic acid sequences. In addition to the three characterized *Arabidopsis* ACP genes, two more ACP isoforms have been identified recently. The sequence of 25 amino acids of the amino terminus for the major leaf-specific *Arabidopsis* ACP isoform has been obtained, and the nucleotide sequence for what might be an *Arabidopsis* mitochondrial ACP isoform, has been characterized (Dave Shintani and John Ohlrogge, personal comm.). Therefore, ACP isoforms in *Arabidopsis* are encoded by a gene family containing at least five ACP genes.

To date, seven ACP genomic clones [*Acl1* and *Acl3* from barley (15), *ACP1* (36), *ACP2* and *ACP3* (25) from *Arabidopsis* and ACP05 and ACP09 genomic clones from *B. napus* (9)] have been sequenced from higher plants. As mentioned earlier *Arabidopsis ACP2* and *ACP3* are linked. Similarly, barley *Acl1* and *Acl2* have been located on the same chromosome, not more than 10 kb apart (15). DNA blot hybridizations indicated that genes encoding spinach ACP-I and ACP-II isoforms may also be linked (44).

All characterized ACP genes have three introns whose positions are almost completely conserved. Two introns (II and III) are in identical positions in the coding region for mature ACPs. The position of intron I in the transit peptide differs by a few amino acids, which is probably a result of the variable length of the ACP transit peptides. Assuming that *Arabidopsis* ACPs have alanine in the first two positions of the mature ACP, intron II is located between the first and the second amino acid. From the known amino acid sequences for either amino terminus or complete mature ACP of spinach ACP-I (24) and ACP-II (33), barley ACP-I and ACP-II (17), and *B.napus* seed ACP (45), ACP isoforms can have either two (spinach ACP-II, barley ACP-I and *B. napus* ACP) or one (spinach ACP-I, barley ACP-II) alanine at the amino terminus. Spinach ACP-I is the only known ACP isoform that has alanine both as the last amino acid in the transit peptide and, as noted before, as the first amino acid of the mature ACP. In addition, since the last three amino acids in the ACP transit peptide seem to be conserved (44), it is likely that *Arabidopsis ACP1* and *ACP2* have alanines at the first two positions of the mature ACP. A similar organization with an intron positioned after the second amino acid of the mature protein has been found in the *rbcS* multigene family (10). Intron III has been found in the middle of the highly conserved region surrounding the phosphopantetheine binding site. Even though in most genes, such highly conserved regions are not interrupted by introns, some members of the chalcone synthase multigene family (31) and the triosephosphate isomerase gene from *Aspergillus nidulans* (28) have an intron in the conserved active site region.

Transit peptides of the *Arabidopsis* ACPs, like other ACP transit peptides, have a high content of hydroxyamino acids. At the carboxy terminus of the transit peptides an amino acid motif (I/V S C) appears to be conserved in the majority of known ACPs. The same amino acid motif was found in some other proteins that are imported into plastids and was proposed to be a consensus sequence for the cleavage site (12). However, upon more detailed analysis of the amino acid sequences of transit peptides of nonhomologous plastid imported proteins, that motif was not found (47).

Even though *Arabidopsis* and *Brassica* ACP transit peptides share a few identical amino acid motifs, the overall low amino acid sequence identity, 35-40%, is similar to the low sequence identities of transit peptides of other plastid imported proteins (20). Heijne et al. (47) have analyzed amino acid sequences of over twenty nonhomologous transit peptides and did not find any of the "homology blocks" reported by Tobin (19). Heijne and coworkers (47) pointed out that the structures of transit peptides differ between stroma-targeted proteins and thylakoid-targeted proteins. Transit peptides of stroma-targeted proteins have three distinct regions. The amino terminal region contains many Pro and Gly residues and the first ten amino acids are mostly uncharged. The central region is rich in Ser and is of variable length. The carboxy-terminal region contains an increased proportion of Arg residues and can form an amphiphilic β-strand. In contrast, transit peptides of proteins targeted to the thylakoids have mosaic-structures. As stroma targeted ACPs, *Arabidopsis* ACP-1, *B. napus* ACP embryo cDNA and spinach ACP-I transit peptides are consistent with these guidelines. However, the transit peptides of other plant ACPs lack

an arginine-rich carboxy terminal region.

The *Arabidopsis ACP2* and *ACP3* genes are very similar (90% at the nucleic acid level) (Table 1). Even their transit peptides are highly identical at both amino acid and nucleic acid levels. The high degree of identity between these two clones indicates that *ACP2* and *ACP3* are result of a very recent gene duplication event. Similarly, coding regions of the *B. napus* and *B. campestris* ACPs have high degrees of identity. Since *B. napus* is an allotetraploid originating from a cross between *B. campestris* and *B. oleracea*, the characterized ACP gene from *B. campestris* is probably the ancestor of the ACP05 gene from *B. napus*. Based on the high sequence identities of *Arabidopsis ACP1* with *B. napus* embryo cDNA ACP (41) and *B. campestris* cDNA ACP (39), the three ACP genes are also closely related.

The 5′ untranslated region of *Arabidopsis ACP1* is G+C rich. In contrast, the 5′ untranslated regions of *Arabidopsis ACP2* and *ACP3* are rich in thymidines (25). Even though the primary structures of the promoter regions of *ACP1* and *ACP2* are different, RNA blot hybridizations appeared to have similar expression patterns for both *ACP1* and *ACP2* genes. However, we should keep in mind that ACP-1 and ACP-2 probes may hybridize to similar ACP messages on RNA blots. Therefore, the differences in the promoter sequences might result in different expression patterns of *ACP1*, *ACP2* and *ACP3*, that could not be detected by standard RNA blot analysis. Similarly to *Arabidopsis*, the two ACP genes from barley, *Acl1* and *Acl3*, have different promoter sequences (47). The proximal region of the barley *Acl3* promoter has a higher G+C content than does that of *Acl1*. In

addition, three GC elements that are very similar to the recognition sequences of the Sp1 factor of RNA polymerase II (30) were found only in *Acl3*. These features lead to the conclusion that the minor leaf ACP isoform, that is encoded by *Acl3* gene, may be a constitutively expressed ACP isoform, similar to the ACP-II isoform from spinach (44).

The positions of the putative RNA polyadenylation consensus sequences (18) in the three *Arabidopsis ACP* genes differ. In *Arabidopsis ACP1*, the putative polyadenylation site is only 24 nucleotides away from the translational stop site. The *ACP2* gene has an identical sequence 220 nucleotides downstream from the stop codon. Two putative sequences, similar to the polyadenylation consensus sequences were found in the *ACP3* gene, 232 and 259 nucleotides 3′ of its translational stop codon. Possible multiple polyadenylation sites were also found in barley *Acl2* and *Acl3* genes (15), as well as in spinach ACP-II (44).

In spite of the small genome size of *Arabidopsis*, ACP isoforms in that crucifer are apparently encoded by a large gene family. In *B. napus*, the gene family of seed-expressed ACP isoforms is estimated to have approximately 35 genes (41). Even when we take into account the fact that *B. napus* is an allotetraploid, the number of ACP genes is still high. In spinach, too, the low number of ACP isoforms identified by immunoblot analysis (24) differs from the information obtained from DNA hybridization studies. Evidence from DNA hybridizations indicates that more than two ACP genes are present in the spinach genome (44), the authors have suggested that some of them may be pseudogenes and/or some may encode

identical proteins.

Arabidopsis ACP-1 and ACP-2 derived nucleic acid probes turned out to be very specific, detecting only ACP genes that are identical (*ACP1* and *ACP2*) or very similar to the probes (*ACP3*). The use of a less specific probe such as the 66-mer (15) that is complementary to the highly conserved region that surrounds the prosthetic group binding site was ineffective with genomic DNA, because of the position of the third intron. In all ACP genes that have been characterized so far, intron III is located in the middle of that highly conserved region. Therefore, under low stringency conditions, the 66-mer hybridized to a large number of fragments, whereas under increased stringency washing conditions, most of the hybrids were melted.

In conclusion, I have characterized two ACP clones, *ACP1* and *ACP2* which have 70% identity at the nucleotide level. DNA blot hybridizations with gene specific probes could detect three ACP genes. However, the use of a less specific probe and continued work by Dave Shintani indicated that *Arabidopsis* ACP isoforms are encoded by a gene family that has more than three genes. The two ACP genes, *ACP1* and *ACP2*, seem to be expressed in leaves, roots and developing seeds. Even though their expression pattern is similar to the constitutive expression pattern of the spinach ACP-II isoform, the possibility that similar but not identical ACP messages were detected could not be ruled out. Additional characterization of the ACP clones combined with the RNase protection analysis of their expression patterns will be necessary for the complete characterization of the ACP

gene family in *Arabidopsis*.

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CHAPTER THREE

CHARACTERIZATION OF *ARABIDOPSIS* ACYL CARRIER PROTEINS

ABSTRACT

Arabidopsis tissues reveal a complex pattern of expression of ACP isoforms. Immunoblot analysis of the leaf, root and seed protein extracts separated by native PAGE and denaturing 1M urea PAGE indicated that each tissue, in addition to the constitutive ACP isoforms, expresses one or more tissue-specific isoforms. The immunoblot identifications of ACP bands were confirmed by acylation of ACP extracts with *E. coli* acyl-ACP synthetase.

Coding regions for the mature protein of the three characterized *Arabidopsis* ACP clones (*ACP1*, *ACP2* and *ACP3*) were expressed in *Escherichia coli*. Recombinant *Arabidopsis* ACPs were partially purified on an ion-exchange DE-52 column. It was shown that each of the three ACPs have different mobilities on polyacrylamide gel electrophoresis. In addition, each of the identified ACP isoforms commigrated with a different *Arabidopsis* ACP isoform expressed in leaves, roots and developing seeds.

Introduction

Acyl carrier proteins are one of the best analyzed components of the fatty acid synthase in plants (20). However, although a large number of cDNA and genomic ACP clones have been characterized to date, only a few ACP isoforms have been identified and correlated with the corresponding DNA sequences. In spinach, ACP-I and ACP-II cDNA clones (30,31) and the mature isoforms (17,22) have been characterized. The ACP-I isoform is a spinach major leaf isoform, whereas ACP-II is a minor isoform. The separation of barley ACP isoforms on DEAE columns (14) and the palmitoylation of ACPs with [^{14}C]palmitate, revealed the presence of the three ACP isoforms (3). For the major leaf ACP-I isoform, and one of the minor leaf isoforms, ACP-II, amino acid sequences have been obtained (14). Recently, three cDNA and two genomic ACP clones were isolated from barley. The cDNA clones encode ACP-I (12), ACP-II (11), and a third isoform, designated ACP-III (12), whose expression has not yet been defined. The two barley ACP genomic clones, *Acl1* and *Acl3*, encode ACP-I and ACP-III isoforms (13). The analysis of 5'untranslated regions of barley *Acl3* revealed several DNA motifs similar to the ones found in mammalian housekeeping genes. Therefore, the authors proposed that the *Acl3* gene might be constitutively expressed as is the case with spinach ACP-II.

Initial work on the characterization of the *Arabidopsis* ACP isoforms (3) revealed a similar pattern of expression that of spinach and barley. In *Arabidopsis* leaves, one major and two minor isoforms were detected by polyclonal antibodies raised against spinach ACP-I. Identical or similar

RNA messages to the *Arabidopsis ACP1* and *ACP2* mRNAs were shown to be expressed in leaves, roots and developing seeds. Because ACP-1 and ACP-2 RNA probes did not cross-hybridize with each other, under the conditions used, described in the Methods section of the Chapter two, it could be concluded that at least two different ACP isoforms were expressed in leaves, roots and seeds. However, only a single ACP isoform was detected by spinach ACP-I antibodies in *Arabidopsis* root protein extracts that were resolved by denaturing SDS PAGE (3). Because of the difference in the number of ACP isoforms present in roots at the RNA level with the number of detected isoforms by spinach ACP-I antibodies, I wanted to use a different system for a detailed analysis of *Arabidopsis* ACP isoforms.

According to Ohlrogge et al. (21), the ACP concentration in spinach leaf chloroplasts was calculated to be approximately 8 μM, which is 10 to 25 times lower than the estimated ACP concentration in *E. coli* (20). Thus, the purification of ACP isoforms from *Arabidopsis* tissues for the amino acid sequencing and for the biochemical characterization of the proteins would require large amounts of the tissue to start with. For that reason, coding regions for mature ACP-1, ACP-2 and ACP-3 were expressed in *E. coli* as a source of the *Arabidopsis* isoforms for the protein analysis. According to Guerra et al. (4), the expression of synthetic spinach ACP-I clone in *E. coli* at levels above that of constitutive *E. coli* ACP did not significantly affect bacterial growth. Similarly, it was expected that *Arabidopsis* ACP isoforms would not affect the growth of bacterial cells either. Therefore, using *Arabidopsis* ACPs expressed in *E. coli* as standards, I wanted to define the isoforms encoded by the three

characterized *Arabidopsis ACP1*, *ACP2* and *ACP3* genes and to determ ine the number of ACP isoforms expressed in protein extracts in different *Arabidopsis* tissues.

Experimental procedures

Material. *Arabidopsis thaliana* (L.) Heynh. var. Columbia plants were grown either in soil or in hydroponic cultures as described in Chapter 1. *E. coli* DH5a strain was used for the subcloning procedures and for the expression of the mature *Arabidopsis* ACPs.

Subcloning and sequencing. Restriction digestion analysis of the plasmids was done according to the enzyme manufacturers' protocols. Plasmid DNA purification, DNA agarose gel analysis and subcloning of the fragments were performed as described in the Methods section of Chapter 1. Sequencing of the generated plasmids was done following the U.S. Biochemical protocol for double stranded DNA sequencing using dideoxy chain termination sequencing method and Sequenase 2.0.

Oligonucleotide purification. Oligonucleotides that were used for polymerase chain reactions (PCR) were desalted on C-18 Sep-Pak Cartridges according to the manufactureres' protocols with some modifications. Cartridges were first rinsed with 10 ml of methanol, then with 10 ml of ddH_2O and finally with 10 ml of 50 mM TEA-Ac. 3 ml of oligonucleotide solution that was prepared by mixing 500 μl of the original

oligonucleotide solution made in ddH_2O, with 2500 μl of 50 mM TEA-Ac, was loaded on the column. Salts were washed off the column with 10 ml ddH_2O, and oligonucleotides were eluted from the column with 3 ml of 50 mM TEA-Ac:methanol (1:1). 1 ml fractions were collected, and the concentration of the oligonucleotide solution was calculated from measurements of the UV absorbance at 260 nm.

Polymerase chain reaction (PCR). PCR was done with plasmid DNA templates (pA1120-E, pJD120, pAD4) using Taq DNA polymerase (Perkin Elmer Cetus) according to the instructions provided by the manufacturer. Initial melting of the DNA template was done at 94°C for 3 minutes. Thirty cycles of 1 minute of melting at 94°C, two minute annealing at 37°C and two minute extension at 72°C, were followed by seven minute complete extension at 72°C. PCR amplified fragments were analyzed in Tris-borate buffer either by 1 - 1.2 % agarose gel electrophoresis or by PAGE.

Expressison of *Arabidopsis* ACP-1, ACP-2 and ACP-3 isoforms in *E.coli*.

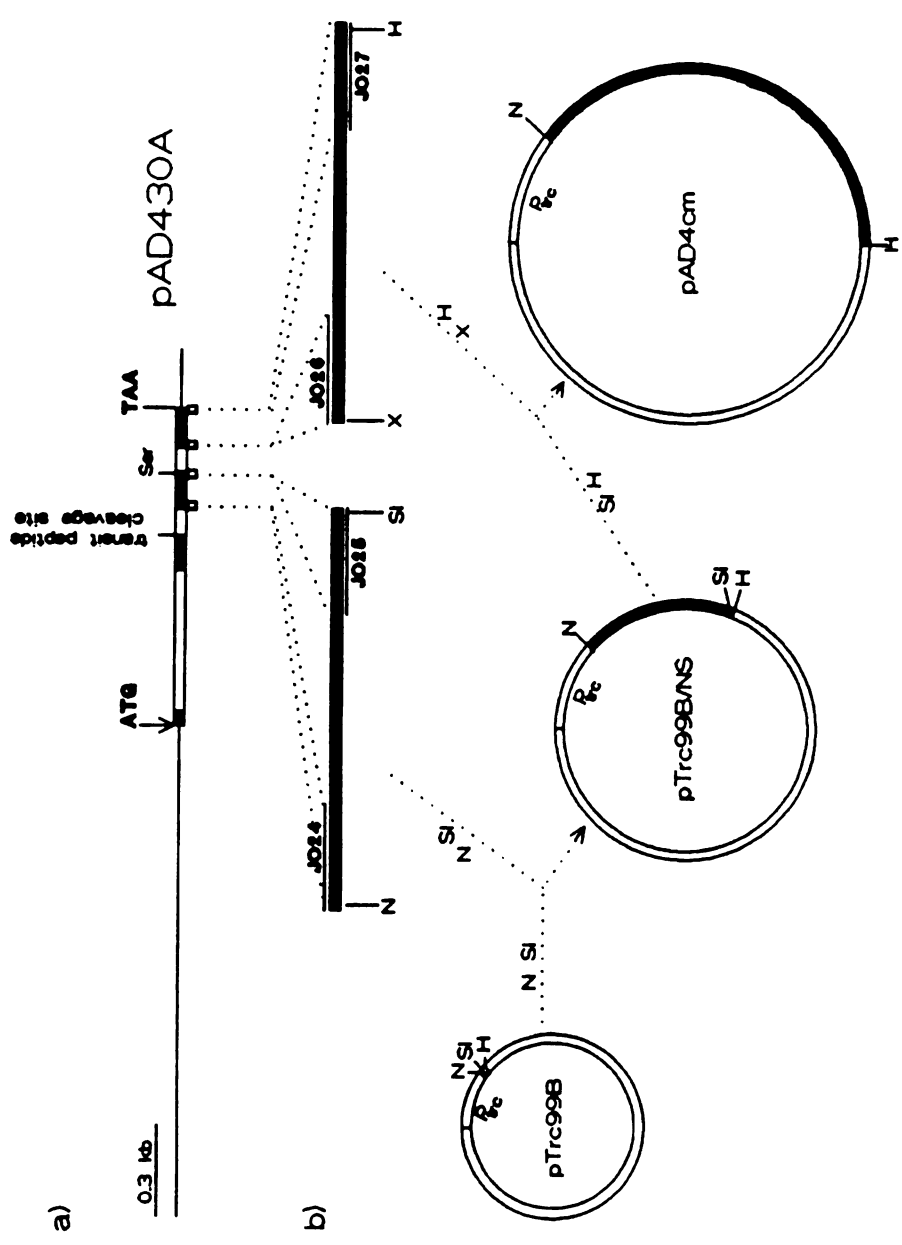
(i) Construction of the expression plasmid with ACP-1 mature coding region. The coding region for mature ACP-1 was produced by PCR-mediated amplification of the third and fourth exons from the genomic clone, pAD4. Four 21-nucleotide oligomers complementary to the 5′ and 3′ regions of the two exons were designed with each carrying a different restriction site (*Nco*I, *Xho*I, *Sal*I or *Hin*dIII). The *Xho*I and *Sal*I sites that were introduced at the 3′ and 5′ ends of the coding regions of the third and fourth exons, respectively, were destroyed following ligation. Codon degeneracy was used to preserve the amino acid sequence of the generated

mature ACP-1, while the two restriction sites were introduced into the coding regions to enable the ligation of the third and fourth exons (Fig. 10). The recombinant fragment was subcloned into the pTrc 99B expression vector (1), and the newly generated plasmid, pAD4cm, was introduced into *E.coli* DH5α strain.

(ii) Construction of the expression plasmid with mature ACP-2 coding region. A 24 nucleotide oligonucleotide and the universal sequencing primer were used as primers to amplify a 0.5 kb fragment encoding mature ACP-2 protein. The 24-mer introduced an *Nco*I site at the codon for the first amino acid of the mature protein. The amplified fragment was subcloned by blunt end ligation into pBS(+) vector. Following digestion with *Nco*I and *Eco*RI, the fragment was excised and subcloned into pTrc 99B expression vector (Fig.11a). The resulting plasmid, pJD120, was introduced into *E.coli* DH5α strain.

(iii) Construction of the expression plasmid with ACP-3 mature coding region. A third ACP gene, A2, described by Lamppa and Jacks (19), differs from *ACP-2* in the mature coding sequence by only a single amino acid. At position 80, ACP-2 has Phe, whereas ACP-3 has Leu. Therefore, site-directed mutagenesis was used to change the amino acid Phe to generate a recombinant plasmid with a coding region for the mature ACP-3. A 22 nucleotide long primer that contained the codon for Leu instead of the codon for Phe at amino acid 80, and universal sequencing primer were used to PCR amplify a *Sac*I/*Eco*RI fragment of the pA1120-E clone. The fragment was then digested with both enzymes and subcloned into the truncated

Figure 10. Schematic representation of the subcloning strategy for expression of mature coding regions of *Arabidopsis* genomic ACP-1 clone in *E. coli*. A) Schematic representation of the structure of *Arabidopsis ACP1* gene. ATG - putative translational start site; Ser - prosthetic group binding site; TAA - translational stop codon. B) Subcloning of the PCR fragments into the pTrc99B expression vector. JO24 = 5'*ttgccatggcggcaaaacaagagacg*3'; JO25 = 5'*atggtcgaccgtgtcgagagaatctg*3'; JO26 = 5'*cagctcgagatagtaatgggtttaga*3'; JO27 = 5'*ttaaagcttaaattacttcttct cgt*3'. RI = *Eco*RI; H = *Hin*dIII; S = *Sac*I; N = *Nco*I; B = *Bcl*II; Sl = *Sal*I; X = *Xho*I. Solid black regions in the map denote coding sequences, white boxes denote intervening sequences, and lines represent 5' and 3' untranslated sequences.


pAD430A
TAA
Ser
transit peptide cleavage site
ATG
0.3 Kb
a)
b)
JO17
JO16
JO14
H
X
Sl
N
pAD4cm
pTrc99BANS
pTrc99B
Ap
R

pJD120 plasmid to replace its original fragment (Fig.11b). The newly generated plasmid, pMZ120L, was introduced into *E.coli* strain DH5α.

The fidelity of the amino acid sequence of the three mature ACP isoforms was confirmed by sequencing portions of the generated clones using the dideoxy chain termination sequencing method.

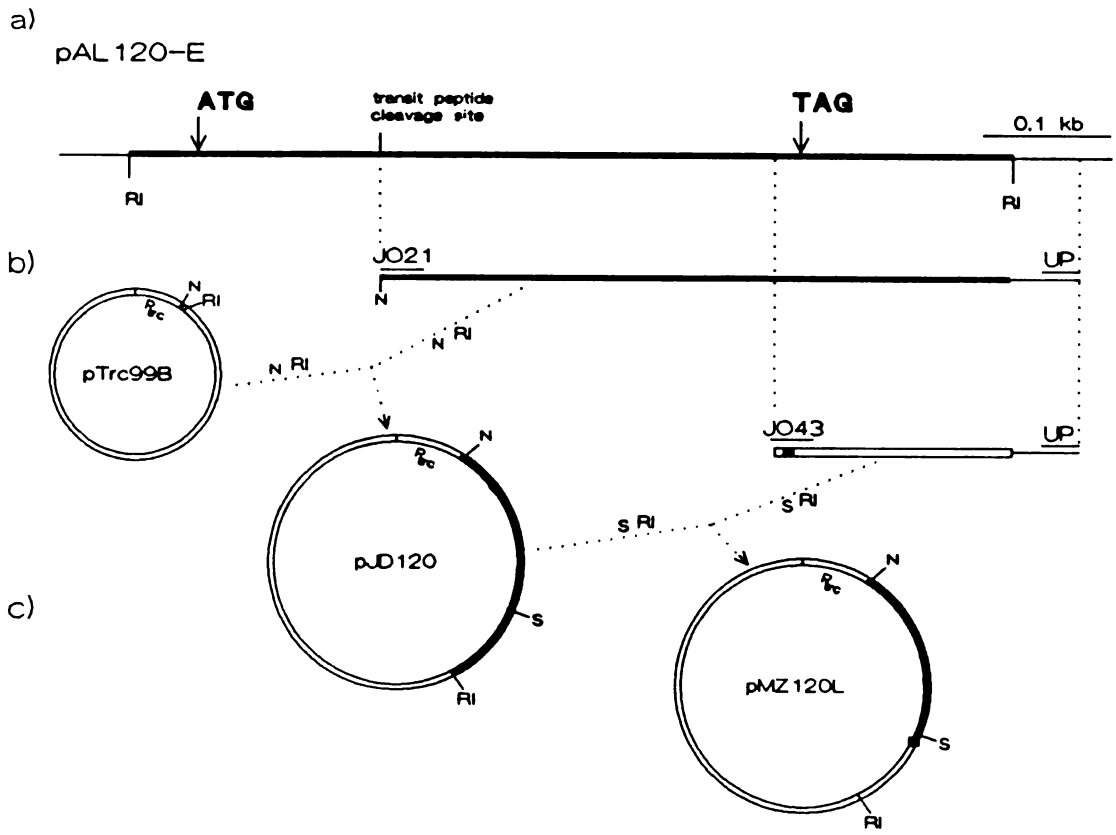
Expression of mature *Arabidopsis* ACP isoforms in *E. coli* and their purification. *E. coli* transformed with either pAD4cm (ACP-1) or pJD120 (ACP-2) was grown at 37^0C in 1 l of LB supplemented with 100 μg/ml ampicillin and 0.5 mM IPTG to OD_{600} of 4.7. ACPs were isolated according to the procedure of Guerra et al. (4) with a few modifications. Cells were collected by centrifugation at 600 rpm for 30 minutes. The cell pellet was resuspended in 10 volumes of homogenization buffer (0.1M Tris HCl, pH 7.0, 20 mM glycine, 1 mM EDTA, 1 mM DTT). After the addition of 0.3 mg of lysozyme, the homogenate was stirred for 2 hours. At the end of the second hour, 1/2 volume of 0.5% (v/v) Triton X-100 was added and the stirring continued. Bacterial DNA was degraded by incubation of the homogenate with 100 μg of DNase for 10 minutes with continued stirring. Following a few bursts with the polytron, a cell free supernatant was obtained by centrifugation at 10,000 rpm for 15 minutes. The supernatant was adjusted to 60% saturation with $(NH_4)_2SO_4$ and left overnight at 4^0C with constant stirring. Precipitated proteins were collected by centrifugation at 10,000 rpm for 15 minutes. Soluble proteins from the supernatant were precipitated by adding TCA to 5% followed by incubation on ice for one hour. After centrifugation at 10,000 rpm for 15 minutes,

the pellet was redissolved in 20 mM MES, pH 6.1, containing 1 mM DTT. The pH was brought to 7.2, and the solution was applied to a DE-52 column which was equilibrated with 20 mM MES, pH 6.1, containing 1 mM DTT. Once the protein extract was applied to the column, the column was washed with the same buffer that was used for its equilibration. Proteins were eluted with 100 ml of a NaCl linear gradient (0 M to 0.5 M) made in 20 mM MES, pH 6.1, containing 1 mM DTT; 1-2 ml fractions were collected. Fractions were assayed for ACP by an acyl-ACP synthetase assay (27).

A 300 ml LB culture of *E. coli* cells transformed with pMZ120L (ACP-3) was induced by 0.5 mM IPTG. The soluble fraction containing ACP-3 isoform was obtained by grinding the cell pellet in 2.5 % TCA (1:5, w/v). Tissue debris and precipitated proteins were removed by centrifugation at 15,000 rpm for 5 minutes. By resuspending the pellet in 50 mM MOPS, pH 6.8, ACP was brought back to solution. The solution was again centrifuged for 5 minutes at 15,000 rpm to remove insoluble material. For deacylation of ACP, the pH of the solution was brought to between 8.5 and 9.0 and DTT was added to 100 mM. The extract was then incubated for 10 minutes at 37^0C. Following precipitation of proteins in 10% TCA, ACPs were dissolved in 50 mM MOPS, pH 6.4-6.8.

Polyacrylamide gel electrophoresis and immunoblot analysis. SDS/PAGE was performed as described by Laemmli (18). Proteins were resolved on a 3% polyacrylamide/0.4% bisacrylamide stacking and 15% polyacrylamide/0.4% bisacrylamide 1.5 mm thick resolving gel in a 0.025 M Tris HCl, pH 9.0, 0.19 M glycine, 10 % (w/v) SDS buffer system. Gels were run at 30 mA/gel

Figure 11. Schematic representation of the strategy for expression of the mature coding regions of *Arabidopsis* ACP-2 (pAL120-E) and ACP-3 in *E. coli*. A) Structure of the pAL120-E cDNA clone. ATG - putative translational start site; Ser - prosthetic group binding site; TAG - translational stop codon. B) Strategy used for the PCR amplification and cloning of the mature ACP-2 coding region and 3' nontranslated region. C) Mature ACP-3 was constructed by replacing the *Sac*I/*Eco*RI fragment from pJD120 with a PCR-amplified *Sac*I/*Eco*RI fragment that contained the codon for Leu instead of the codon for Phe (marked with a bold letters in the sequence of the primer and with a star in the figures). JO21 = 5'*ccatgggctgcaaaacctgagaca*3; JO43 = 5'*tgaggagctcttgttggaaaag*3'; UP = 5'*actggccgtcgttttac*3'; RI = *Eco*RI; H = *Hin*dIII; S = *Sac*I; N = *Nco*I.


a)
pAL120-E
ATG
transit peptide cleavage site
TAG
0.1 kb
RI
RI
b)
JO21
UP
N
N
RI
pTrc99B
N RI
JO43
UP
S RI
pJD120
S
RI
c)
pMZ120L
S
RI

for 5 hours. Prior to loading, ACP samples were reduced with 50 mM DTT.

Native PAGE and denaturing urea PAGE were prepared according to Rock & Cronan (26). A 13% polyacrylamide/0.4% bis(acrylamide) resolving gel was overlayered with 3% polyacrylamide/bis(acrylamide) stacking gel. Urea gels were a modification of native PAGE. 1M urea was included both in the stacking and in the resolving gel as well as in an sample buffer. Samples were deacylated in 100 mM DTT at pH 8.5 - 9.0, and ACPs were blocked with 20 mM NEM. Gels were run in a 0.025 M Tris HCl, pH 9.0, 0.19 M glycine buffer system at 30 mA/gel for 3 hours.

The gels were electrophoretically transblotted to 0.2 um nitrocellulose filter in a 39 mM glycine, 48 mM Tris HCl, pH 9.0, 20% (v/v) methanol system at 0.8 mA/mm^2 of the gel surface. Proteins were fixed onto the filter with a 3 minute incubation in 5% (w/v) TCA, followed by thorough washing in dH_2O.

Nitrocellulose was blocked with Blotto for 2 hours. Spinach ACP-I antibodies were added to the fresh TBST buffer in a dilution of 1:500. Filters were immunoblotted for one hour. The primary antibody solution was poured off and filters were washed three times with TBST for 10 minutes each. Secondary, goat anti(rabbit IgG)-alkaline-phosphatase conjugated antibodies (1:2,000), were added to fresh blocking solution and incubated for one hour. At the end of the incubation, the antibody solution was poured off and filters were washed three times in TBST for 10 minutes each. Immunostaining was carried out in alkaline phosphatase

buffer with NBT and BCIP dissolved in 70% and 100% N,N-dimethylformamide, respectively. Color development was stopped by thorough rinsing in ddH_2O.

Preadsorbing of the antibodies with *E. coli* extract. A 25 ml liquid culture of *E. coli* cells was inoculated from a single cell colony and left to grow overnight at 37^0C with constant shaking. Cells were then pelleted at 6,000 rpm for 10 minutes. The pellet was resuspended in 1 ml PBS, pH 7.0 and incubated in a boiling water bath for 10 minutes. Following a 5 minute sonication, the insoluble material was pelleted by centrifugation at 13,000 rpm for 5 minutes. 1 ml of the supernatant was mixed with 20 ml of 1:500 primary anti-spinach ACP-I antibodies for 40 minutes before use.

Radiolabeling od ACPs. *Arabidopsis* ACP isoforms that were expressed and purified from *E. coli*, as well as fractions that were assayed for ACPs, were acylated with [^{14}C]palmitate (56 mCi/mmol) in a reaction catalyzed by an acyl-ACP synthetase (27). ACPs from the *Arabidopsis* leaf, root and seed material were labeled with uniformly labeled [U-^{14}C]palmitate (>500 mCi/mmol). The enzyme was partially purified from *E. coli* cells by John Shanklin according to Rock and Cronan (27). The reaction mix contained 0.1 M Tris HCl, pH 8.0, 10 mM $MgCl_2$, 0.4 M LiCl, $3x10^5$ DPM [^{14}C]-16:0, 5 mM ATP, 5 mM DTT, sample and acyl-ACP synthetase. After one hour incubation at 37^0C, half of the reaction mix was spotted onto DE 81 filter discs that were left to dry in a hood. Unincorporated label was washed off the filters with three changes of 80% isopropanol, 20% phosphate saline buffer [10 mM KPO_4, pH 6.0, 0.1 M NaCl]. Following the addition of the scintillation fluid, radioactivity was counted in a Beckman scintillation

counter and the acylated ACPs were resolved by native PAGE.

RESULTS

Previous studies have shown that polyclonal spinach ACP-I antibodies could detect one and three ACP isoforms in *Arabidopsis* root and leaf protein extracts, respectively, that were resolved in an SDS\PAGE system (3). The analysis of protein extracts from *Arabidopsis* developing seeds with anti-spinach ACP-I antibodies detected a single ACP isoform commigrating with a root and with a minor leaf ACP isoform (Fig. 12). Therefore, based on the SDS\PAGE immunoblot analysis, *Arabidopsis* tissues appeared to have three ACP isoforms. However, it was noticed that mobilities of the ACPs in SDS\PAGE were affected by the content of charged and hydrophobic amino acids in the mature ACP (20), suggesting that different isoforms with a similar percentage of hydrophobic, acidic and basic residues would comigrate in that gel system. In addition, the ACP isoforms that differ in a single amino acid, like *Arabidopsis* mature ACP-2 and ACP-3 isoforms (19), would probably not be separated by SDS/PAGE.

Native polyacrylamide gels developed by Rock and Cronan (26) and denaturing urea PAGE were shown to have high resolving power for the intermediates of fatty acid synthesis (24). The immunoblot analysis of protein extracts from *Arabidopsis* leaf, root and developing seed tissues that were resolved in either native/PAGE or urea PAGE indicated that each tissue has a minimum of four ACP isoforms (Fig. 13,14).

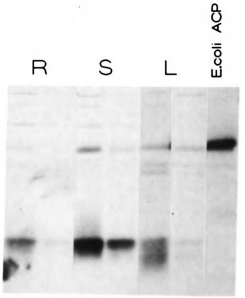


Figure 12. Immunoblot analysis of ACP isoforms in *Arabidopsis* root, seed and leaf protein extracts. Crude protein extracts were resolved in SDS-15%PAGE. Blots were probed with antiserum to spinach ACP-I. L = *Arabidopsis* leaf extract; R = *Arabidopsis* root extract; S = *Arabidopsis* seed extract.

Surprisingly, leaves, roots and seeds had at least three comigrating ACP isoforms, indicating that the genes encoding these isoforms might be constitutively expressed. In addition, tissue-specific ACP isoforms were detected in leaves, roots and seeds. The relative abundance of different ACP isoforms varied in different tissues. The most abundant isoform in leaves appeared to be the tissue-specific isoform. However, in roots, all four detected putative ACP isoforms were relatively equally abundant, whereas in seeds the three "constitutive" ACP isoforms were more abundant than the ones that were expressed in a tissue-specific manner.

Even though both *ACP1* and *ACP2* mRNAs appeared to be expressed in leaves, roots and developing seeds, their deduced amino acid sequences could not reveal the identity of the isoforms they were encoding. Therefore, as an initial step in defining the protein products that will enable analysis of different functions of ACP isoforms in *Arabidopsis,* I have expressed mature ACP-1, ACP-2 and ACP-3 isoforms in *E. coli*. For the expression of mature ACP-1, PCR-mediated amplification of the third and the fourth exons was followed by their sequential subcloning into the *E. coli* expression vector, pTrc 99B, yielding pAD4cm (Fig. 10). The coding region for the mature ACP-2 was PCR-amplified and subcloned into the same vector, yielding pJD120 (Fig. 11a). Site-directed mutagenesis was used for the change of the amino acid Phe at position 80 of the ACP-2, into the amino acid Leu of the ACP-3. A DNA fragment from pJD120, that contained a codon for Phe, was replaced by a PCR amplified fragment that contained a codon for Leu, yielding pMZ120L (Fig. 11b). Fidelity of the final subcloning products was confirmed by sequencing portions of the fragments.

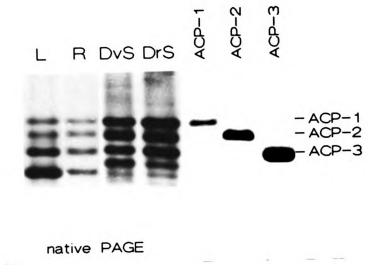


Figure 13. Immunoblot analysis of ACP isoforms in *Arabidopsis* root, seed and leaf protein extracts. Crude protein extracts were separated using native PAGE. Blots were probed with antiserum to spinach ACP-I. L = *Arabidopsis* leaf extract; R = *Arabidopsis* root extract; DvS = *Arabidopsis* developing seed extract; DrS = *Arabidopsis* dry seed extract; ACP-1 = purified *Arabidopsis* ACP-1 standard; ACP-2 = purified *Arabidopsis* ACP-2 standard; ACP-3 = *E. coli* extract containing expressed *Arabidopsis* ACP-3 isoform.

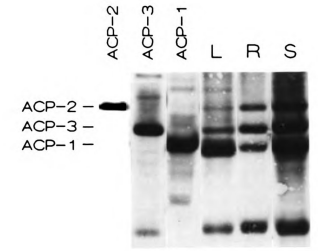


Figure 14. Immunoblot analysis of ACP isoforms in *Arabidopsis* root, seed and leaf protein extracts. Crude protein extracts were separated using denaturing 1M urea PAGE. Blots were probed with anti-spinach ACP-I antibodies. L = *Arabidopsis* leaf extract; R = *Arabidopsis* root extract; S = *Arabidopsis* dry seed extract; ACP-1 = purified *Arabidopsis* ACP-1 standard; ACP-2 = purified *Arabidopsis* ACP-2 standard; ACP-3 = *E. coli* extract containing expressed *Arabidopsis* ACP-3 isoform.

E. coli DH5α cells were transformed with one of the generated plasmids and after induction with 5 mM IPTG the recombinant *Arabidopsis* ACPs were expressed 1.5 to 3.3 fold over the basal level of expression of the *E. coli* ACP. The *Arabidopsis* ACP-1 and ACP-2 isoforms were purified from the *E. coli* ACP on a DE-52 column in a 0 to 0.5 M NaCl gradient. The ACP activity in different fractions was measured using an acyl-ACP synthetase (EC 6.2.1.) assay (27). Because the elution profile did not reveal any information on the nature and origin of particular isoforms, samples from the fractions with ACP activity were separated in SDS/PAGE and identified by immunoblotting. Identical filters were immunobloted with either spinach ACP-I antibodies or with the same antibodies that were blocked with *E. coli* extract. In both cases, immunoblots revealed that *Arabidopsis* ACP-1 (Fig. 15) and ACP-2 isoforms (data not shown) eluted from the DE-52 column with a lower salt concentrations than *E. coli* ACPs. *E. coli* expressed *Arabidopsis* mature ACP-1 was eluted with a higher salt concentration than the *Arabidopsis* ACP-2 (0.3 vs 0.22 M NaCl), whereas *E. coli* ACPs were eluted with a similar salt concentrations in both purifications (0.35 and 0.4 M NaCl) (Fig. 16 and 17).

Mature *Arabidopsis* ACP-1, ACP-2 and ACP-3 isoforms that were purified from the *E. coli* cells were used as standards in the protein analysis of the *Arabidopsis* ACP isoforms. The three ACP isoforms were easily resolved on both native PAGE and 1M urea PAGE. Even the ACP-2 and ACP-3 isoforms that differ by only a single amino acid at position 80, were separated from each other (Fig. 13 and 14). The mature ACP-1, ACP-2 and ACP-3 proteins were found to commigrate with three putative ACP isoforms detected in

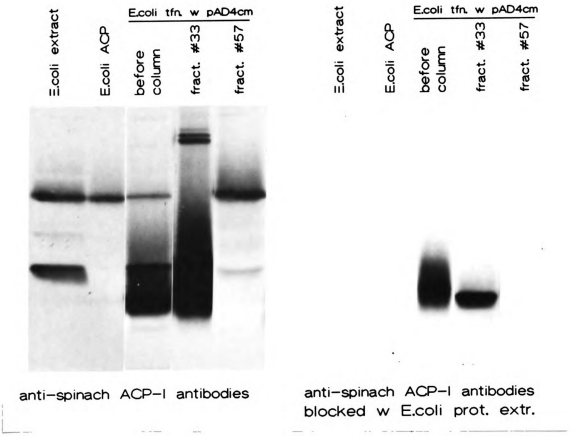


Figure 15. Immunoblot analysis of the column fractions from the purification of *Arabidopsis* ACP-1 expressed in *E. coli*. Proteins were separated by SDS-PAGE and ACPs were detected with anti-spinach ACP-I antibodies that were either preblocked with *E. coli* protein extracts or were not preblocked. Due to the high salt concentration migration of the *Arabidopsis* ACP-1 in fraction #33 was slightly altered.

Figure 16. Purification of *Arabidopsis* ACP-1 expressed in *E. coli*. Protein extracts of IPTG induced *E. coli* cells that were transformed with pAD4cm, were applied to a DE-52 ion exchange column. 1 ml fractions were collected and assayed for ACP using [^{14}C]palmitate and *E. coli* acyl-ACP synthetase. The elution peaks of the *Arabidopsis* ACP-1 and *E. coli* ACP are indicated by the arrows.

Arabidopsis ACP-1 purification on DE-52 column

Acyl ACP synthetase assay

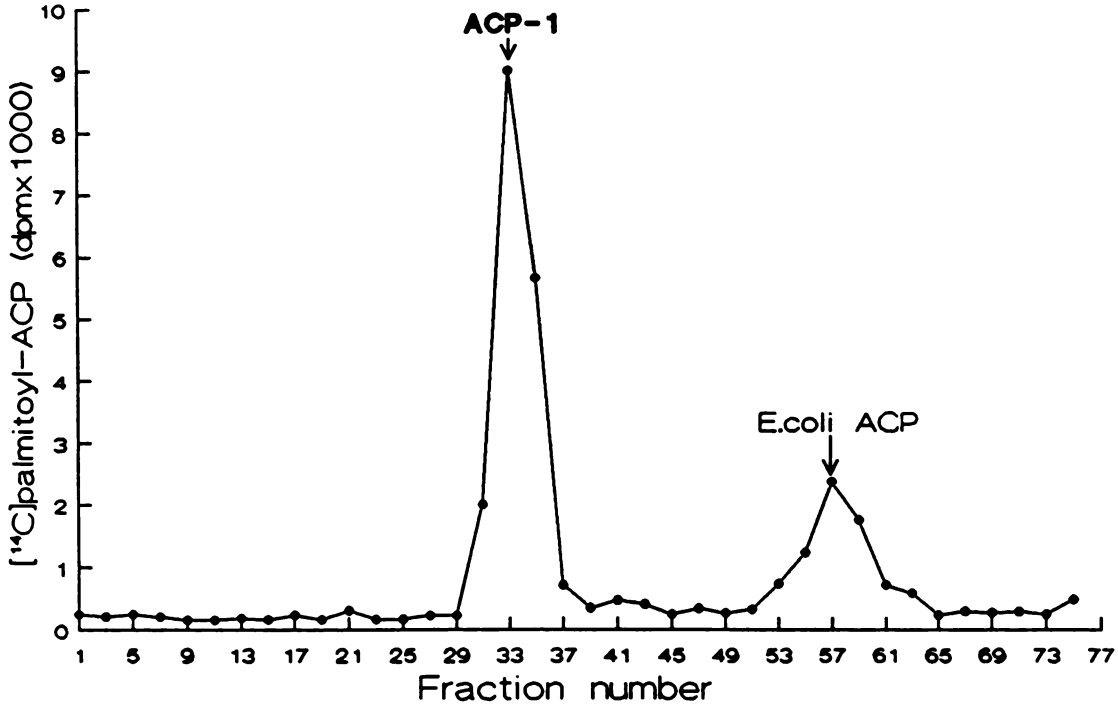
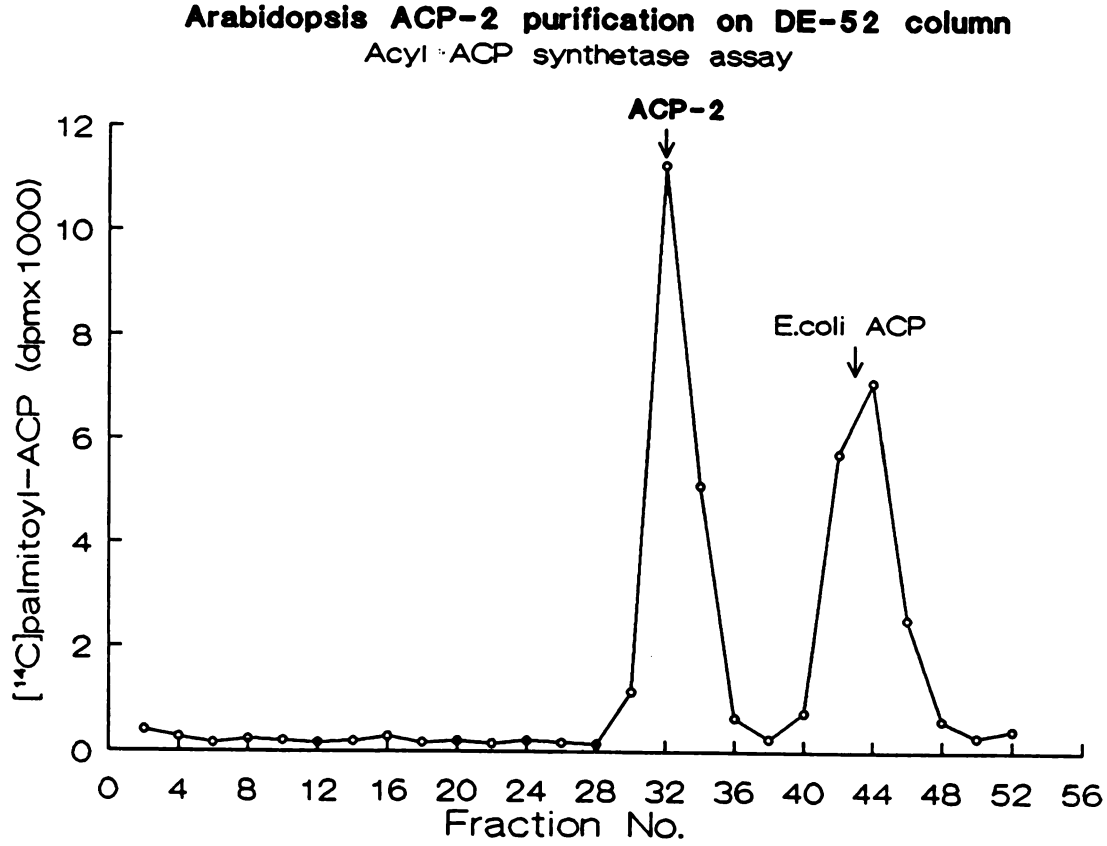


Figure 17. Purification of *Arabidopsis* ACP-2 expressed in *E. coli*. Protein extracts of IPTG induced *E. coli* cells that were transformed with pJD120, were applied to a DE-52 ion exchange column. 4 ml fractions were collected and assayed for ACP using [^{14}C]palmitate and *E. coli* acyl-ACP synthetase. The elution times of the *Arabidopsis* ACP-2 and *E. coli* ACP are indicated by the arrows.


Arabidopsis ACP-2 purification on DE-52 column
Acyl ACP synthetase assay
ACP-2
E.coli ACP
[14C]palmitoyl-ACP (dpmx1000)
12
10
8
6
4
2
0
0 4 8 12 16 20 24 28 32 36 40 44 48 52 56
Fraction No.

immunoblots of protein extracts of *Arabidopsis* leaves, roots, developing and dry seeds, indicating that *ACP1*, *ACP2* and *ACP3* genes are expressed in a constitutive manner. Based on relative abundances, ACP-1, ACP-2 and ACP-3 isoforms appear to be three of the four more abundant isoforms in seeds, whereas in leaves they are less abundant isoforms.

Some of the proteins that were detected by polyclonal spinach ACP-I antibodies in immunoblots might possibly be cross-reacting non-ACP proteins. In order to further evaluate the number of ACP isoforms in *Arabidopsis* tissues, ACPs were specifically labeled using *E. coli* acyl ACP synthetase (27) and [^{14}C]palmitate. ACP-enriched leaf, root and seed protein fractions were acylated and the proteins were separated on native PAGE. Proteins were transferred to nitrocellulose membranes and following autoradiography, putative ACPs on the blot were detected by spinach ACP-I antibodies. The autoradiogram showed that *Arabidopsis* leaf, root and seed tissues have expressed multiple ACP isoforms, some of which are tissue-specific (Fig. 18). A smaller number of ACP isoforms detected on an autoradiogram compared to the number of putative deacylated ACP isoforms on the immunoblots, could be a result of the changes in the mobilities that occur upon acylation of ACPs (27). The acyl group is presumably positioned in the hydrophobic cleft made by three a helical regions of the ACP (15). Upon acylation the conformation of the ACP changes, resulting in the changed mobility on native PAGE.

The [^{14}C]palmitoylated ACP-1 was separated on native PAGE from the [^{14}C]palmitoylated ACP-2 isoform (Fig. 18). The ACP-2 isoform was found

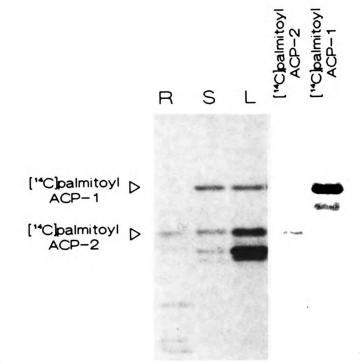


Figure 18. Palmitoylation of *Arabidopsis* ACPs in root, dry seed and leaf protein extracts. ACP-enriched crude protein extracts were acylated using [^{14}C]palmitate and *E. coli* acyl-ACP synthetase. Acyl-ACPs were separated by native PAGE. Proteins were blotted to nitrocellulose membrane and exposed to a film for 1 week. L = *Arabidopsis* leaf extract; R = *Arabidopsis* root extract; S = *Arabidopsis* dry seed extract; [^{14}C]palmitoyl ACP-1 = *Arabidopsis* [^{14}C]-palmitoyl-ACP-1 standard; [^{14}C]palmitoyl ACP-2 = *Arabidopsis* [^{14}C]-palmitoyl-ACP-2 standard.

in all three tissues, whereas the ACP-1 isoform was found in leaves and seeds. We cannot rule out the possibility that, due to poorer isolation or acylation of ACP-1, that ACP1 was not recovered as efficiently from the root extracts as from the other tissues. An alternative explanation is that an isoform other than ACP-1 which is expressed in roots comigrates with deacylated ACP-1 on native PAGE. Such an isoform upon palmitoylation, might undergo slightly different conformational change from the ACP-1 and be resolved from it upon native PAGE.

As a result of the analysis of the expression of ACP isoforms in *Arabidopsis* tissues, several constitutive and tissue-specific ACP isoforms were detected by anti-spinach ACP-I antibodies and by autoradiography in leaves, roots and seeds. Based on the labeling studies with acyl-ACP synthetase and on the immunoblot analysis, ACP-2 is clearly expressed in a constitutive manner. Similarly, ACP-1 and ACP-3 seem to be constitutively expressed isoforms. However, due to the complex pattern of ACP isoforms expressed in *Arabidopsis* tissues, for the complete characterization of their expression patterns, labeling studies at the protein level and the analysis of the expression of each isoform at the mRNA level using specific probes will need to be done.

DISCUSSION

In this study *Arabidopsis* mature ACP isoforms, ACP-1 (23), ACP-2 (this study, 19) and ACP-3 (19) and their expression patterns have been

identified. Because of the low abundance of ACPs in plant tissues (21), instead of purifying ACP isoforms from *Arabidopsis* tissues, coding regions for the mature ACPs have been expressed in *E. coli* cells. Protein extracts from *Arabidopsis* leaf, root and seed material and purified *E. coli* expressed *Arabidopsis* ACPs were analyzed by the combination of PAGE, immunoblotting and radiolabeling studies.

Rock and Cronan (26) reported that *E. coli* ACP comigrates in SDS/PAGE with proteins of MW 20,000, even though its calculated MW is 8,847. They pointed out that the highly acidic nature and the low number of hydrophobic residues of *E. coli* ACP probably decrease the efficiency of binding of SDS. As a result, the protein migrated more slowly than one would expect from its molecular weight. A similar effect was reported for plant ACP isoforms. For example, barley ACP-I of MW 9,500, comigrates with *E. coli* ACP on SDS/PAGE (14), whereas spinach ACP-I (MW 8708) and ACP-II (MW 8,899), comigrate with proteins of MW 14,500 and 16,000 (17,22), respectively. However, mature *Arabidopsis* ACP-1 (MW 9,159) and ACP-2 (MW 8,962) isoforms, comigrate with proteins of MW similar to their estimated molecular weight. The differences in the behaviour of ACPs on SDS/PAGE might be in the ratio of acidic and basic amino acids. *E. coli* ACP has more acidic and fewer basic amino acid residues than either *Arabidopsis* ACP-1 or ACP-2 (20 vs 18 acidic and 7 vs. 11 or 13 basic).

Initially, three ACP isoforms that were detected on immunoblots of leaf, root and seed protein extracts resolved by SDS/PAGE (3, Fig. 12) suggesting that *Arabidopsis* might have a small ACP gene family. However,

a minimum of seven putative ACP isoforms were separated in the protein extracts of these tissues in native and denaturing urea/PAGE (Fig. 13 and 14). Even though the resolving power of native and urea PAGE systems was such that ACP isoforms that differ in a single amino acid were resolved, we still cannot exclude the possibility that some ACP isoforms with conservative amino acid changes comigrated. Because of the stability of ACPs and of the preparation of the protein samples multiple bands detected by spinach ACP-I antibodies were probably not a result of the proteolysis of ACP isoforms. As in *Arabidopsis*, SDS/PAGE analysis of the *B. napus* seed protein extract revealed only a single ACP band (33). However, Safford et al. characterized eight *B. napus* ACP cDNA clones, encoding six different ACPs (29). Even though no information on the abundance of any of these isoforms is available, the results suggest that six *B. napus* seed ACP isoforms commigrate in SDS/PAGE system. In addition to *Arabidopsis*, *B. napus*, *Avena sativa* and *H. vulgare* were found to have more than two ACP isoforms expressed in leaf tissue (3). Even some non-vascular plants such as *Polytrichum* and *Marchantia*, were reported to have a minimum of four and six ACP isoforms in leaf tissues, respectively (3). Therefore, the occurence of more than two ACP isoforms does not appear to be restricted only to the Brassicaceae family. To date, spinach is the only higher plant that has two ACP isoforms that are resolved by native/PAGE, by SDS/PAGE and by urea/PAGE (17,22,24). There, ACP-I and ACP-II isoforms were found in leaves, whereas only ACP-II was found in roots and seeds. However, work of Schmid and Ohlrogge (31) indicated that more than two genes might be encoding ACP-I and ACP-II isoforms. As they pointed out, the apparent simplicity at the protein level, might not be represented at

the gene level, where different members of the spinach ACP gene family could be differently regulated by spatio-temporal factors.

Based on the immunoblot analysis of the protein extracts resolved either by native or by denaturing urea PAGE, *Arabidopsis* ACP isoforms encoded by *ACP1*, *ACP2* and *ACP3* appear to be expressed in a constitutive manner. Specific labeling of ACPs with *E. coli* acyl-ACP synthetase and [^{14}C]palmitate in leaf, root and seed protein extracts confirmed the presence of the ACP-2 isoform in all these tissues. Similarly, a recent report of the expression pattern of the b-glucuronidase reporter gene, which was under the control of the ACP-2 promoter in transformed tobacco detected the *ACP2* gene promoter driven expression of GUS in every tissue (2).

Polyclonal anti-spinach ACP-I antibodies, that were used in this study, have poorer cross-reactivity with prokaryotic and monocotyledonous ACPs than with dicotyledonous ACPs (9). Therefore, some of the epitopes on the *Arabidopsis* ACP isoforms are not recognized by the antibodies. However, by using saturating levels of the antibodies in the immunoblot analysis, the intensities of the immunoblot signals represent approximate relative abundances of the ACP isoforms. Hence, in *Arabidopsis* leaves, the three constitutively expressed ACP isoforms are less abundant than the leaf specific isoforms, similarly to the constitutive spinach ACP-II isoform. In contrast to spinach, *Arabidopsis* appears to have isoforms that are specifically expressed even in roots and seeds.

Gene families are common in the plant kingdom (25). Isoforms and isozymes can be localized either in the same (6, 32) or in different cellular compartments (7). While the functional importance of the isoforms in different cellular compartments generally lies in performing similar functions that have been compartmentalized, the functions of different isoforms localized within the same organelle are still unknown. ACP isoforms appear to have a complex distribution within plant cells. Spinach ACP-I and ACP-II (21) and barley ACP-I and ACP-II (14) are found in chloroplasts. ACP-like proteins have been detected in mitochondria (5). Even though *in vitro* data, indicated that oleoyl-ACP acyltransferase and glycerol-3-P acyltransferase are differentially efficient with two spinach ACP isoforms, ACP-I and ACP-II (10), no *in vivo* evidence to date supports that hypothesis. The predictions of the secondary structures of known ACP isoforms reveal very similar arrangements of the a helical and b turn regions. Moreover, between 54% and 64% of both ACP-I and ACP-II pools occur in a nonesterified form (24). The small variations in the secondary structures of ACP isoforms might still be reflected in a different effectiveness with components of the fatty acid synthase. At the same time, ACP isoforms that are located in the same cellular compartment might still be capable of substituting for each other in the reactions of fatty acid and lipid biosynthesis. In this case the presence of a large ACP gene family, as well of the other components of fatty acid synthase, might permit plasticity in the expressional patterns in a spatial and/or temporal manner.

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CHAPTER FOUR

SUMMARY AND PERSPECTIVES

Summary

A characterization of several *Arabidopsis* ACP isoforms at both the DNA and protein level presented in this dissertation can be summarized as follows:

1) A genomic ACP clone encoding the ACP-1 isoform was isolated using *B. napus* cDNA ACP clone as a source of a heterologous probe.

2) The structure of the *Arabidopsis* ACP gene was determined by comparing the cDNA ACP sequences from *B. napus* and *B. campestris* with the *Arabidopsis ACP1* gene. The *Arabidopsis ACP1* gene encodes a protein that has a 54 amino acid long transit peptide and an 83 amino acid long mature protein. The gene contains one intron in the transit peptide coding region and two introns in the coding region for the mature ACP.

3) A cDNA ACP clone that encodes the *Arabidopsis* ACP-2 isoform was isolated from an expression library using anti-spinach ACP-I antibodies.

4) The *ACP2* cDNA clone encodes a 134 amino acid long ACP that has a 52 amino acid long transit peptide.

5) The ACP gene family in *Arabidopsis* has a minimum of three ACP genes.

6) RNA blot analysis indicated that *Arabidopsis* ACP-1 and ACP-2 might be constitutively expressed. In addition, a minimum of two ACP

isoforms are expressed in leaves, roots and seeds.

7) It was shown that native PAGE and urea PAGE should be preferred systems for the analysis of *Arabidopsis* ACP isoforms because of their better resolving power than SDS PAGE. Even ACP isoforms that differ by a single amino acid, like ACP-2 and ACP-3, were resolved in either native or urea PAGE systems.

8) *Arabidopsis* mature ACP isoforms encoded by *ACP1*, *ACP2* and *ACP3* genes are expressed in a constitutive manner.

9) Based on the immunoblot and radiolabeling studies *Arabidopsis* leaf, root and seed tissues each have expressed tissue-specific ACP isoforms.

Perspectives

Plant fatty acid synthase provides fatty acids for the cell membrane synthesis, for the synthesis of cuticular waxes in epidermis and for the synthesis of storage lipids in developing seeds (6,10). While maintenance of cell membranes requires constitutive activity of the components of fatty acid synthase, biosynthesis of waxes and storage lipids are tissue-specific and/or developmentally regulated events.

To date, multiple isoforms or isozymes have been identified for some of the components of fatty acid synthase. One and two isozymes of the malonyl-CoA:ACP transacylase, that were expressed in a tissue-specific manner, have been found in soybean seed and leaf tissues, respectively

(3). Very recently, three dimeric forms of the β-ketoacyl ACP synthase I, composed of two different polypeptides, have been detected in barley leaves (9). Multiple isoforms of a protein cofactor, ACP, that are expressed in a tissue-specific manner in higher plants, have been reported even in simple multicellular algae (1). It seems likely that isoforms/isozymes of some or of all components of plant fatty acid synthase have different activities in reactions of fatty acid and some steps of lipid biosynthesis, although direct *in vivo* evidence for this possibility is lacking.

The work presented in this dissertation initiated characterization of an ACP gene family in *Arabidopsis*. ACP nucleotide sequences for *ACP1* and *ACP2* genes will allow thorough characterization of the expression patterns of ACP isoforms in *Arabidopsis* at the cellular level, using either *in situ* hybridizations or the analysis of the expression of a reporter gene driven by either one of the promoters. The sequences also provide information on the suitable approach that needs to be undertaken for the identification of other members of ACP gene family.

Very little is known about the regulation of the expression of fatty acid synthase in plants. Sequence information for the promoter regions of the two characterized ACP genes in *Arabidopsis* will permit initial analysis of the regulatory elements, that can be extended as additional ACP genes are identified. In order to perform these experiments one should look for the expression patterns of chimeric genes comprised of different deletions of the promoter regions fused to the coding region of reporter genes (such as

β - glucuronidase).

Recently, several groups have reported the involvement of ACP in reactions other than fatty acid and lipid biosynthesis in prokaryotes, such as activation of prohaemolysin (4) and synthesis of membrane derived oligosacharides (11). ACP has been characterized as a subunit of an NADH:ubiquinone oxidoreductase in both *N. crassa* (8) and in bovine heart mitochondria (7). However, its function in the respiratory complex is not known. Different ACP isoforms have been differentially efficient with the enzymes at the branching point in the initial steps of glycerolipid biosynthesis *in vitro* (2). Therefore, in order to address the question of the functional importance of ACP isoforms in plant metabolism, with a special emphasis on fatty acid and lipid biosynthesis, the expression of particular ACP isoforms should be inhibited by expressing antisense RNA ACP constructs. Identification of the ACP isoforms as provided in this thesis, encoded by the *ACP1*, *ACP2* and *ACP3* genes will enable easy detection of the inhibited isoforms by antibodies. *Arabidopsis* plants with an almost complete inhibition of one or more of the ACP isoforms can be analysed in detail. In addition, the plants carrying two or three different ACP antisense clones could be obtained by crossing the transformants that have a single ACP antisense clones. This way, it will be possible to analyse the effects of the inhibition of either all constitutive and/or tissue-specific ACP isoforms.

Availability of the expression clones with coding regions for the three mature *Arabidopsis* ACP isoforms provides an opportunity to analyze the

secondary structure of an ACP. The site-directed mutagenesis of R-spinach ACP-I isoform confirmed that Ser 38 is a phosphopantetheine binding site (5). Similarly, site-directed mutagenesis of other regions of ACP isoforms in combination with the deletional analysis of central, amino and carboxyl terminal regions might provide an information on the functional importance of different areas of ACP in the interaction with components of fatty acid synthase.

Arabidopsis, like *Neurospora*, seems to have a mitochondrial ACP isoform (Dave Shintani person. commun.). Availability of the clones for the choloroplast-targeted and mitochondrial-targeted ACP isoforms will provide tools for the analysis of the transport mechanism of the isoforms into different organelles. In addition, it will be possible to tackle the question of the cellular location of the attachment of the prosthetic group to ACP.

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APPENDIX

APPENDIX

ACP nomenclature

In spinach, the leaf-specific ACP isoform is designated as ACP-I and the constitutive ACP isoform as ACP-II (1). Barley ACP-I and ACP-II isoforms appear to be expressed in leaves (2). However, no information is available on the expression of these two isoforms in other tissues. Because of the apparent similarities in the nomenclature with acid phosphatases, characterized barley ACP genes were named *Acl1* and *Acl3* (3). The analysis of ACP isoforms in *Arabidopsis* revealed the probable presence of more than 6 ACP isoforms.

Therefore, we suggest that the following nomenclature be applied to specify genes and isoforms for acyl carrier proteins in *Arabidopsis* in order to: (i) avoid confusion because the complete information on the expression patterns is not available yet for *Arabidopsis* ACP genes and (ii) to establish an easy system for their naming since the number of ACP isoforoms in *Arabidopsis* is high. Following these guidelines, each ACP isoform should be labeled with "ACPn", where n is an Arabic number. The number should define the ACP isoform according to time of publication of either nucleic acid or amino acid sequence. (By numbering different isoforms using Arabic numerals instead of Roman numerals we will avoid correlation with spinach and barley isoforms in the terms of their

expression patterns and will provide a simpler system for the numerization of large number of isoforms). Wild type genes should be labeled in upper case italics, mutant genes in lower case italics and protein isoforms in capital letters. According to these guidelines, the first published ACP gene from *Arabidopsis* (4) would be *ACP1*, its mutant would be *acp1* and the isoform encoded by that gene ACP-1.

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