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# Expression of Holo and Apo Forms of Spinach Acyl Carrier Protein-I in Leaves of Transgenic Tobacco Plants

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**Acyl carrier protein (ACP) is a chloroplast-localized cofactor of fatty acid synthesis, desaturation, and acyl transfer. We have transformed tobacco with a chimeric gene consisting of the tobacco ribulose-1,5-bisphosphate carboxylase promoter and transit peptide and the sequence encoding the mature spinach ACP-I. Spinach ACP-I was expressed in the transformed plants at levels twofold to threefold higher than the endogenous tobacco ACPs as determined by protein immunoblots and assays of ACP in leaf extracts. In addition to these elevated levels of the holo form, there were high levels of apoACP-I, a form lacking the 4'-phosphopantetheine prosthetic group and not previously detected *in vivo*. The mature forms of both apoACP-I and holoACP-I were located in the chloroplasts, indicating that the transit peptide was cleaved and that attachment of the prosthetic group was not required for uptake into the plastid. There were also significant levels of spinach acyl-ACP-I, demonstrating that spinach ACP-I participated in tobacco fatty acid metabolism. Lipid analyses of the transformed plants indicated that the increased ACP levels caused no significant alterations in leaf lipid biosynthesis.**

## INTRODUCTION

Acyl carrier protein (ACP) is an essential cofactor for at least a dozen enzymes of plant lipid biosynthesis, including the multiple enzyme system of fatty acid synthase (FAS), stearoyl-ACP desaturase, oleoyl-ACP hydrolase, and two acyl-ACP transferases (Ohlrogge, 1987). Virtually all of the ACP in spinach mesophyll cells is localized in the chloroplasts (Ohlrogge, Kuhn, and Stumpf, 1979). Acyl carrier proteins are nuclear-encoded and are synthesized as precursors (Ohlrogge and Kuo, 1984b) with transit peptides that are cleaved as the mature proteins are transported into the chloroplasts. In developing seed and leaf, the levels of both ACP and its mRNA are positively correlated with the rates of fatty acid synthesis (Ohlrogge and Kuo, 1984a; Hannapel and Ohlrogge, 1988). There are multiple isoforms of ACP in the leaves of higher plants (Hoj and Svendsen, 1984; Kuo and Ohlrogge, 1984a). The most abundant form in spinach leaf is designated ACP-I. The amino acid sequences for acyl carrier proteins have been determined for several prokaryotes (Cooper, Boyce, and Lueking, 1987; Hale, Jordan, and Leadlay, 1987) and for both monocotyledonous and dicotyledonous plants (Hansen, 1987; Rose et al., 1987; Post-Beittenmiller, Hlousek-Radojcic, and Ohlrogge, 1989), and have been shown to be highly conserved. The most conserved region includes the attachment site for the 4'-phosphopantetheine pros-

thetic group, which is attached via a phosphodiester linkage to a serine residue. During fatty acid synthesis, desaturation, and acyl transfer, the fatty acid is covalently bound to the 4'-phosphopantetheine group on ACP via a thioester linkage. The transfer of the prosthetic group from CoA to apoACP is catalyzed by holoACP synthase. Recent results indicate that this enzyme is located in the cytoplasm, and therefore pre-apoACP is converted to pre-holoACP prior to its transport into plastids (Elhoussein, Miernyk, and Ohlrogge, 1988).

The stoichiometric relationships of plant acyl carrier protein and the enzymes of FAS are not known. However, there is speculation that they may exist in an ordered complex. If this is true, the expression of all the FAS proteins may be coordinately regulated. Since the nature of the organization and regulation of FAS is unknown, we are interested in the consequences of altering the levels of ACP on lipid biosynthesis and expression of endogenous ACPs. To overexpress ACP, we transformed tobacco leaf discs with a chimeric gene in which the coding sequence for spinach ACP-I is regulated by the tobacco ribulose-1,5-bisphosphate carboxylase (rubisco) small subunit promoter (Mazur and Chui, 1985). In this study we examined the levels of spinach apoACP-I, holoACP-I, and acyl-ACP-I resulting from expression of the chimeric gene in transformed tobacco plants, and investigated whether the expression of spinach ACP-I affected either the endogenous tobacco ACP levels or *in vivo* lipid metabolism.

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## RESULTS

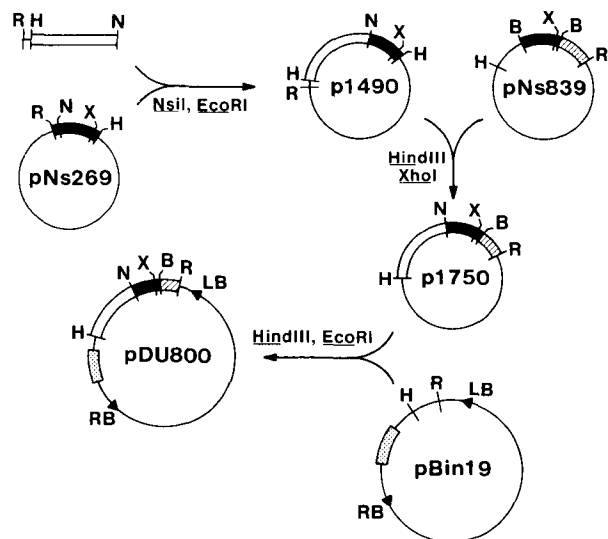
### Construction of pDU800

The rubisco small subunit (*rbcS*) promoter and transit peptide were ligated to the sequence encoding mature spinach ACP-I, as shown in Figure 1. We chose the *rbcS* promoter to obtain a high level of transcription that could be regulated by light. The *rbcS* transit peptide was required to facilitate targeting of the mature spinach ACP-I protein into the chloroplasts because the ACP-I synthetic gene (Beremand et al., 1987) used in these experiments does not include a transit peptide. The polyadenylation sequence from the nopaline synthase gene (*nos*) was ligated 3' to the *rbcS*:ACP-I fusion, and this construct was further subcloned into pBin19 (Bevan, 1984). The resulting plasmid, pDU800, was introduced into *Agrobacterium tumefaciens*, which was subsequently used to infect tobacco leaf discs. Transformed plants were selected by kanamycin resistance (*kan<sup>R</sup>*). The pDU800-transformed and pBin19-transformed (control) plants were indistinguishable in appearance from each other and from wild-type tobacco.

### Analyses of ACPs by Protein Immunoblots

Proteins from leaf sections of eight independently isolated *kan<sup>R</sup>* pDU800-transformed plants were separated on SDS or native polyacrylamide gels, blotted to nitrocellulose, and probed with antibodies raised against spinach ACP-I. The polyclonal antibodies prepared against spinach ACP-I cross-react with ACPs of other plants (Kuo and Ohlrogge, 1984b) and therefore detected both spinach and tobacco ACP in the protein immunoblots. Typical results from one transformed (pDU800) and one control (pBin19) plant are shown in Figure 2. In control plants (lanes 6, 7, and 8), three major bands were detected on blots from the native gel (A) and four bands on blots from the SDS gel (B). Because tobacco ACPs have not previously been characterized, these bands were preliminarily designated ACP isoforms based on their cross-reactivity with antibodies against spinach ACP-I and mobilities characteristic of ACPs in the two gel systems. In addition to these bands, blots of plants transformed with pDU800 revealed additional major bands, one of which co-migrated with the mature spinach ACP-I standard in both gel systems. This indicated that the *rbcS*:spinach ACP-I gene was expressed, and, furthermore, that the transit peptide was cleaved. Processing of the *rbcS*:spinach ACP-I chimeric protein suggested that it was correctly targeted to tobacco chloroplasts.

To distinguish further between the tobacco and spinach forms of ACP, protein immunoblots of native gels were also probed with a polyclonal antibody preparation that was first preadsorbed with tobacco leaf extracts. In these



**Figure 1.** Construction of a Chimeric Gene Using the Tobacco *rbcS* Promoter and Transit Peptide Sequences and the Spinach ACP-I Mature Sequence.

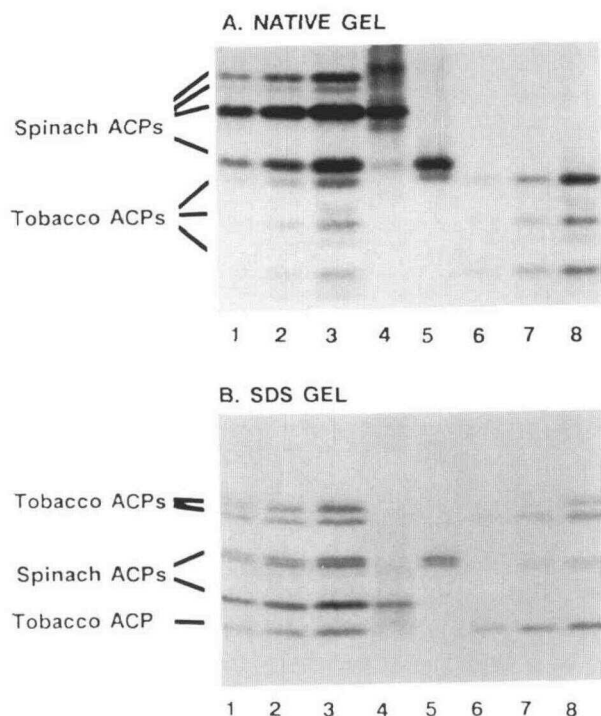
An EcoRI, NsiI fragment containing the tobacco *rbcS* promoter and transit peptide was inserted into EcoRI, NsiI-digested p1490. To provide a polyadenylation sequence, p1490 was digested with HindIII and XhoI, and the *rbcS*:ACP fragment was inserted into pNs839, containing the *nos* polyadenylation sequence. The chimeric gene contained on a HindIII, EcoRI fragment was then inserted into the polylinker region of pBin19. B, H, N, R, and X refer to BamHI, HindIII, NsiI, EcoRI, and XhoI restriction endonucleases, respectively. LB and RB refer to left and right border repeats in the T-DNA regions of pBin19. □ *rbcS*, ■ ACP, ▨ polyA+, □ neomycin phosphotransferase (*kan<sup>R</sup>*).

experiments the bands identified as tobacco ACPs in Figure 2A were eliminated, and the bands identified as spinach ACPs remained (see Figure 6), confirming that they were derived from the *rbcS*:spinach ACP-I gene product.

All eight independent *kan<sup>R</sup>* plants analyzed had readily demonstrable levels of spinach ACP-I. Furthermore, the level of spinach ACP-I expression did not vary significantly among the eight transformants. Preliminary experiments indicated that the antibodies reacted with equal sensitivity to tobacco and spinach ACPs such that the relative levels of tobacco and spinach ACPs in the transformed plants could be estimated by the intensities of the protein immunoblot signals. Based on densitometric scans of the blots, the abundance of spinach ACP-I appeared to be twofold to threefold higher than the sum of the tobacco ACPs.

### Identification of ApoACP-I in Transformed Plants

In addition to the mature ACP-I, a second major band unique to the pDU800-transformed plants was detected in



**Figure 2.** Protein Immunoblot Analyses from Native and SDS-Polyacrylamide Gel Electrophoresis Show That the Level of Spinach ACP-I Is Twofold to Threefold Higher than That of Tobacco ACPs.

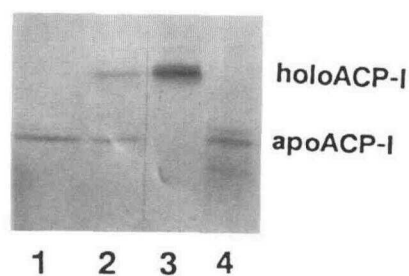
Aliquots (2  $\mu$ L, 5  $\mu$ L, or 10  $\mu$ L) of leaf homogenates prepared from tobacco plants transformed with pDU800 (lanes 1 to 3) or control plants transformed with pBin19 (lanes 6 to 8) were electrophoresed on either a native gel or an SDS gel, and the gels were processed for protein immunoblot analyses. Purified spinach apoACP-I and holoACP-I were included as standards (lanes 4 and 5, respectively).

(A) Native gel. Lanes 1 to 3, the relative mobilities ( $R_f$ ) for the spinach ACPs are: 0.49, 0.50, 0.54, and 0.62. Lanes 4 and 5, the spinach ACP standards (lanes 4 and 5) have  $R_f$  values of 0.54 (apoACP-I) and 0.62 (holoACP-I). Lanes 6 to 8, the tobacco ACPs have relative mobilities of 0.65, 0.71, and 0.78.

(B) SDS gel. Lanes 1 to 3, the spinach ACPs have  $R_f$  values of 0.67 and 0.73, which correspond to the holoACP-I and apoACP-I standards in lanes 5 and 4, respectively. Lanes 6 to 8, the tobacco ACPs have  $R_f$  values of 0.56, 0.59, and 0.78. Prestained molecular weight markers (Bethesda Research Laboratories) of 13.7 kD, 8.1 kD, and 2.7 kD had  $R_f$  values of 0.58, 0.74, and 0.79, respectively.

apoACP-I band, the protein was isolated from a native gel and tested as a substrate in spinach holoACP synthase assays. In these assays, the 4'-phosphopantetheine prosthetic group is donated by CoA to apoACP-I, converting apoACP-I to holoACP-I. The reaction mixtures were then separated on an SDS gel, and the apo and holo forms were visualized by protein immunoblot analyses. The results are shown in Figure 3. Prior to treatment with holoACP synthase (lane 1), there was a single ACP band that co-migrated with the spinach apoACP-I standard (lane 4). After treatment with holoACP synthase (lane 2), an additional band that co-migrated with the spinach holoACP-I standard band (lane 3) was produced. This demonstrated that the protein was a substrate for spinach holoACP synthase and was therefore the apo form of ACP. In addition, its reactivity with anti-spinach ACP-I preparations preadsorbed with tobacco leaf extracts indicates that it was spinach apoACP-I.

Two additional, less intensely stained bands that migrated slightly above the spinach apoACP-I standard were detected only in native gels of pDU800-transformed plants (Figure 2A, lanes 1 to 3). These were determined to be spinach ACPs based on their reactivity with anti-spinach ACP-I that had been preadsorbed with tobacco leaf extract (see Figure 6). To establish whether they were holo or apo forms of spinach ACP, the differential sensitivity of these forms to reducing agents was exploited. When holoACP-I was run on protein gels without a reducing agent, its mobility was retarded, presumably due to oxidation of the sulfhydryl on the 4'-phosphopantetheine prosthetic group. However, the mobility of apoACP-I was not affected. When extracts from pDU800 transgenic plants were run on native gels without a reducing agent, the mobility of the holoACP-I



**Figure 3.** Identity of the Putative ApoACP-I Band Was Confirmed by Demonstrating That It Was a Substrate for HoloACP Synthase.

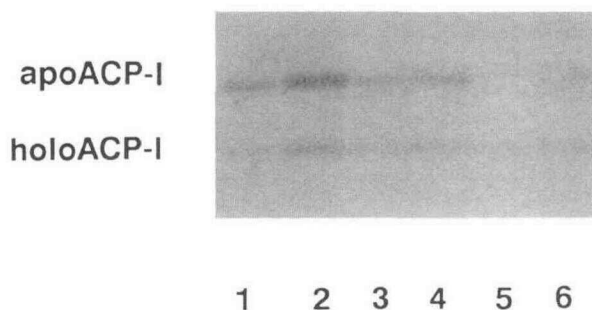
Proteins co-migrating with an apoACP-I standard were eluted from a preparative native gel, concentrated, and tested as substrate for spinach holoACP synthase. The holoACP synthase reaction mixture was loaded onto an SDS gel, and the ACPs were visualized by protein immunoblot analysis. Lane 1, the sample prior to treatment with holoACP synthase; lane 2, the sample after treatment with holoACP synthase; lane 3, spinach holoACP-I standard; lane 4, spinach apoACP-I standard.

protein immunoblots. This band co-migrated with a spinach apoACP-I standard in the two different gel systems (see Figure 2; compare lanes 1 to 3 with lane 4), suggesting that it represented spinach apoACP-I. However, the apo form of ACP has not previously been detected in plants (Ohlrogge, 1987). To confirm the identity of the putative

band was slowed, while the mobilities of the apoACP-I as well as the two minor bands were not affected (data not shown). This suggested that the minor bands lack the 4'-phosphopantetheine prosthetic group. It is possible that these minor bands represent incomplete or improper processing of the transit peptide from the *rbcS*:ACP fusion protein. It is unlikely that they represent completely unprocessed precursors because they are not visible on SDS gels (Figure 2B), which resolve the precursor and mature forms of holoACP (Ohlrogge and Kuo, 1984b).

### Chloroplastic Location of the Spinach ACP-I

Observation of the mature forms of spinach apoACP-I and holoACP-I without the transit peptide suggested that they were localized in chloroplasts. To confirm the subcellular location of the spinach ACPs, chloroplasts from transformed tobacco plants were prepared on Percoll gradients. Chloroplasts were either left untreated, treated with trypsin, or lysed and then treated with trypsin. The chloroplast proteins were then separated on a native gel and transferred to nitrocellulose for visualization of ACPs by protein immunoblot analysis, as shown in Figure 4. Both spinach apoACP-I and spinach holoACP-I were present in intact chloroplasts before and after trypsin treatment (lanes 1 to 4), but absent from lysed, trypsinized chloroplast preparations (lanes 5 and 6), as expected for proteins located within the plastids. The chloroplastic location of the spinach holoACPs and apoACPs and their co-migration with standards of the mature proteins suggest that addition of



**Figure 4.** Chloroplast Localization of Spinach ApoACPs and HoloACPs.

Intact chloroplasts were prepared on Percoll gradients and either left untreated, treated with trypsin, or lysed and treated with trypsin. Aliquots (20  $\mu$ L and 50  $\mu$ L) of each treatment were run on a native gel and then processed for immunoblot analysis. Both spinach apoACP-I and holoACP-I were present in the untreated chloroplasts (lanes 1 and 2) and were protected from trypsin in the intact chloroplast preparations (lanes 3 and 4) but were degraded in chloroplast preparations that had been lysed (lanes 5 and 6).

the prosthetic group to ACP is not essential for uptake or processing of the precursor forms.

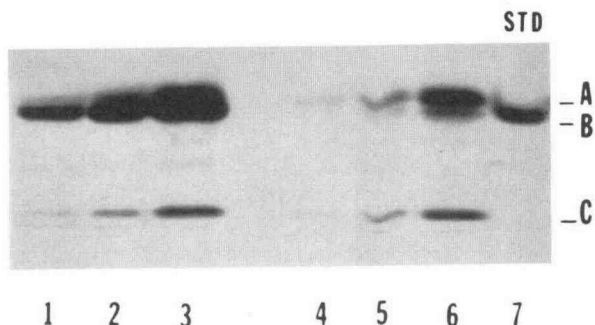
### Activity of Spinach ACP-I in Vitro

Although spinach ACP-I was properly processed and localized in the chloroplasts, it was not known whether the spinach holoACP-I produced in tobacco was functional. One measure of active ACP is in vitro acylation. Therefore, total ACPs were prepared from leaf homogenates of pDU800-transformed and pBin19-transformed (control) F1 plants and then acylated using  $^3$ H-palmitate (C16:0) and *Escherichia coli* acyl-ACP synthetase. Assay of ACP levels by this method indicated that the pDU800-transformed plants contained threefold to fourfold higher total ACP on a fresh weight basis. To examine the endogenous tobacco ACPs and to confirm that the increase in ACP level was due to spinach ACP-I, the  $^3$ H-palmitate-labeled ACPs were resolved on native gels. Aliquots based on gram fresh weight of leaf were separated, and the acylated ACPs were visualized by fluorography, as shown in Figure 5. The major tobacco acyl-ACP (band A, lanes 4 to 6) does not co-migrate with the spinach acyl-ACP-I standard (lane 7). However, one of the minor tobacco acyl-ACPs co-migrates with spinach acyl-ACP-I (compare band B, lanes 6 and 7). In pDU800-transformed plants (lanes 1 to 3), band B represented the major acyl-ACP species, while band A remained relatively unchanged. The increased intensity of band B was therefore due to in vitro acylation of spinach ACP-I. A comparison of the relative band intensities by scanning densitometry again indicated that the level of spinach holoACP-I was twofold to threefold higher than the level of the endogenous tobacco ACPs. This was in agreement with the direct ACP assay and with the relative levels of spinach and tobacco ACPs as estimated from densitometric scans of protein immunoblots. Furthermore, when the levels of the endogenous tobacco ACPs in pDU800 and pBin19 plants were compared on a gram fresh weight basis, there were no significant differences, indicating that production of spinach ACP-I did not significantly alter tobacco ACP expression.

### Acylation of Spinach ACP-I in Vivo

In spinach leaves that are actively synthesizing fatty acids, approximately 5% to 20% of the endogenous acyl carrier protein is esterified with acyl groups of eight carbons or longer (J.F. Battey and J.B. Ohlrogge, unpublished results). These acyl groups can be removed by treating leaf extracts with 0.1 M DTT at pH 9.0. To determine whether the spinach ACP-I produced in tobacco was acylated in vivo, leaf extracts from pDU800 and pBin19 transgenic plants were either loaded directly onto native gels or deacylated prior to loading. Duplicate gels were run and the proteins





**Figure 5.** Spinach HoloACP-I Expressed in Tobacco Can Be Acylated in Vitro.

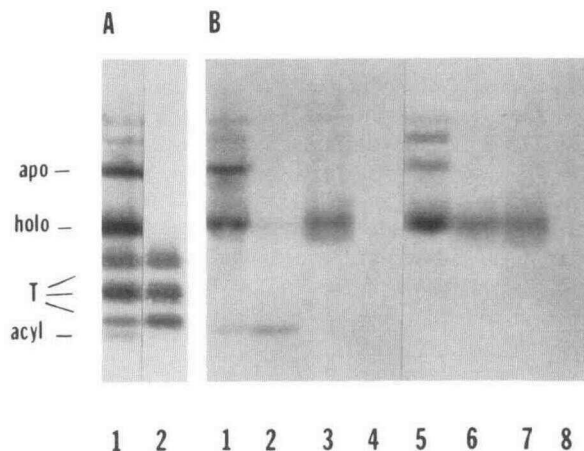
Total ACPs prepared from leaf homogenates from ACP-I transformed and pBin19 control plants were acylated using *E. coli* acyl-ACP synthetase and separated on native gels. The acyl-ACPs were visualized by fluorography. The aliquots of extracts used in the acylation assays were based on gram fresh weight equivalents. Lanes 1, 2, and 3 are 5-μL, 10-μL, and 25-μL aliquots of acyl-ACPs from ACP-I-transformed extracts, respectively. Lanes 4, 5, and 6 are 5-μL, 10-μL, and 25-μL aliquots of acyl-ACPs from control extracts, respectively. Lane 7 is purified spinach acyl-ACP-I standard, 30,000 dpm. Band A is the major tobacco acyl-ACP. Band B (lanes 4 to 6) is a tobacco acyl-ACP that co-migrates with the spinach standard (lane 7). In lanes 1 to 3, band B becomes the most intense band with relatively little change in the intensity of band A. Band C in lanes 1 to 6 is *E. coli* acyl-ACP, which occurs as a contaminant in the *E. coli* acyl-ACP synthetase preparation (Cooper et al., 1989).

were transblotted to nitrocellulose. One blot was processed normally and the other was processed with polyclonal antibody that had been preadsorbed with tobacco extract to distinguish between tobacco and spinach ACPs. In Figure 6A, pDU800 (lane 1) and pBin19 (lane 2) extracts are shown with normal immunoblot processing. Tobacco ACPs (T bands) and spinach apoACP-I and holoACP-I are all clearly visible. In the pDU800 extract (Figure 6A, lane 1), there is an additional lower band, which co-migrates with the spinach acyl-ACP-I standard (Figure 6B, lane 2). In lane 1 of the preadsorbed immunoblot (Figure 6B), this band is still visible, while the tobacco ACPs are not, thus indicating that this band is a form of spinach ACP. Under deacylating conditions, this band disappears with a concomitant increase in the intensity of the spinach holoACP-I band (Figure 6B, lane 5). Similarly, the standard acyl-ACP-I band visible in lane 2 disappears under deacylating conditions with a concomitant appearance of holoACP-I (lane 6). Protein immunoblots of pBin19 extracts processed with preadsorbed antibody have no detectable ACP bands (Figure 6B, lanes 4 and 8). Therefore, the presence of a band that co-migrates with standard acyl-ACP-I, reacts with preabsorbed antibody, and disappears under deacylating conditions together indicated that a portion of spinach holoACP-I expressed in tobacco was acylated in vivo.

In protein immunoblots from heavily loaded native gels, faint bands were detected in both the pBin19 control and pDU800 plants that appeared to correspond to tobacco acyl-ACPs based on migrations similar to tobacco acyl-ACPs detected from fluorographs. These bands were severalfold less intense than spinach acyl-ACP-I from comparable loadings (data not shown). Therefore, we conclude that, in addition to increased apoACP-I and holoACP-I levels, the pool of acyl-ACPs has also been increased in pDU800-transformed plants.

#### Analysis of ApoACP-I and HoloACP-I after Transfer of pDU800 Plants to the Dark

Since the spinach ACP-I coding sequences were under the control of the tobacco *rbcS* promoter, shifting light-



**Figure 6.** Spinach ACP-I Is Acylated in Vivo.

A leaf sample from an ACP-I-transformed plant was homogenized in PBS, 10 mM DTT. Half of the extract was left untreated and the other half was deacylated by treatment with 100 mM DTT at pH 9.0. Spinach acyl-ACP-I standard was treated similarly. Duplicate samples were loaded onto two native gels and transblotted to nitrocellulose. One blot was processed normally and the other blot was processed with polyclonal antibody that had been preadsorbed with tobacco extract. Both blots were developed with the same secondary conjugated antibody and color reagents.

(A) Normal immunoblotting processing. Lane 1, extract from DU800 (ACP-I)-transformed plant; lane 2, extract from Bin19 plant. (B) Preadsorbed antibody. Lanes 1 to 4, samples prepared without deacylation; lanes 5 to 8, samples prepared under deacylating conditions; lanes 1 and 5, extracts from DU800 plants; lanes 2 and 6, spinach acyl-ACP-I standard; lanes 3 and 7, spinach holoACP-I standard; lanes 4 and 8, extracts from Bin19 plants. When preadsorbed antibody was used (B), only spinach ACPs are visualized; therefore, the ACPs detected in (B) are spinach ACPs. Under deacylating conditions the lowest band disappears, and there is a concomitant increase in the intensity of spinach holoACP-I band (compare lanes 1 and 5).

grown plants to the dark should reduce transcription of the chimeric *rbcS:spinach ACP-I* gene and allow us to assess the relative stability of apoACP-I and holoACP-I. Plants grown in a growth chamber with six to eight leaves were placed in a light-tight box. Leaf samples were collected at various times, and the ACPs were analyzed by protein immunoblots (data not shown). After 6 days in the dark, the levels of both spinach apoACP-I and holoACP-I had decreased less than 25%. These relatively small decreases in holoACP and apoACP occurred with similar if not identical rates, such that the relative levels of the two spinach ACPs did not change significantly.

### Fatty Acid and Acyl Lipid Composition in Transgenic Plants

Considering the many functions of ACPs in plant lipid synthesis, perturbation of ACP levels might be expected to affect lipid composition. Several studies have reported that shorter (C8-C14) chain length fatty acids are produced when increasing levels of ACP are added to *in vitro* fatty acid synthesis reactions (Huang and Stumpf, 1971; Slabas, Roberts, and Ormesher, 1982; Singh, Nee, and Pollard, 1984). Fatty acids and representative glycerolipids of leaves from transgenic plants were therefore examined.

As shown in Table 1, plants overexpressing ACP exhibited no major changes in overall steady-state fatty acid composition or lipid to dry weight ratios. Short-chain fatty acids remained only minor components of both bulk lipid (Table 1) and individual species, including the triacylglycerols (data not shown). To determine whether short-chain fatty acids were synthesized but failed to accumulate, leaf discs were labeled with  $^{14}\text{C}$ -acetate for 20 min and 60 min,

and individual fatty acids were separated by argentation and reverse-phase thin-layer chromatography. No differences in short-chain fatty acids were detected between control plants and those producing spinach ACP-I. After 20 min of incorporation, most label was found in palmitate (16:0), oleate (18:1), and linoleate (18:2), with trienoic acids (16:3 and 18:3) becoming major species after the 60-min incubation.

Finally, individual lipid classes of pBin19 and pDU800 isolates were separated by thin-layer chromatography in polar and nonpolar solvent systems. No pDU800-dependent changes were apparent when lipids were visualized with iodine. Phosphatidylglycerol, monogalactosyldiacylglycerol, and phosphatidylcholine, lipids that are representative products of plastidial and nonplastidial lipid biosynthetic pathways, were transesterified for gas-liquid chromatography analyses. Within each class, fatty acid profiles of control and pDU800 species were virtually indistinguishable (data not shown).

### DISCUSSION

One of the most striking results of these studies was the presence of spinach apoACP-I. ApoACPs have not previously been detected in plants. However, in eight independently isolated ACP-I-transformed tobacco plants, we found levels of spinach apoACP-I that were similar to those of spinach holoACP-I, i.e., twofold to threefold above the levels of the endogenous ACPs.

The transit peptide was processed and both the apoACP-I and holoACP-I were located in the chloroplast. Previously we have shown that holoACP synthase, which adds the 4'-phosphopantetheine prosthetic group to apoACP, is a cytosolic enzyme (Elhussein, Miernyk, and Ohlrogge, 1988). This observation raised the question of whether prosthetic group attachment was required or involved in the transport of ACP across the organelle envelope. In the case of yeast cytochrome *c*, transport into the mitochondrial inner membrane requires the heme prosthetic group (Dumont, Ernst, and Sherman, 1988). However, the processing of the transit peptide and the location of apoACP-I in chloroplasts indicate that attachment of the prosthetic group is not required for ACP transport into the chloroplast.

An alternative explanation for the chloroplastic location of apoACP-I in these plants is that only pre-holoACP-I was transported into chloroplasts, but a portion was subsequently converted to apoACP-I by a holoACP hydrolase analogous to that found in *E. coli* (Vagelos and Larrabee, 1967). However, our attempts to detect such an activity in spinach have been unsuccessful. Furthermore, the apparent absence of tobacco apoACPs from ACP-I-transformed plants also argues against this interpretation. In addition, the rates at which apoACP-I and holoACP-I levels de-

**Table 1.** Leaf Fatty Acid Composition of Transformed Tobacco Plants

Fatty Acid	DU800	Control
12:0 <sup>a</sup>	0.3 ± 0.1 <sup>b</sup>	0.3 ± 0.1
14:0	1.7 ± 0.2	1.7 ± 0.3
16:0	15.1 ± 1.2	14.1 ± 1.1
t3-16:1	3.2 ± 0.2	3.1 ± 0.4
16:3	7.8 ± 1.0	7.1 ± 0.8
18:0	1.8 ± 0.2	2.1 ± 0.3
18:1	3.6 ± 0.5	3.2 ± 1.2
18:2	14.2 ± 1.7	15.2 ± 3.1
18:3	52.2 ± 2.3	53.1 ± 3.7
Total fatty acid (mg/g, dry wt)	49 ± 4.0	50 ± 5.0

<sup>a</sup> Fatty acids are identified by the number of carbons: number of double bonds. t3-16:1 is *trans*-3-hexadecenoic acid.

<sup>b</sup> Values are weight percent ± SD (n = 15). Four replicate analyses were performed on four F1 plants of each group.

creased in pDU800 plants placed in the dark were very similar. If the holo form were converted to an apo form during degradation, an altered apo to holo ratio might have been expected. For these reasons, it seems unlikely that the spinach apoACP-I arose through the action of a chloroplastic holoACP hydrolase.

Although apoACP is completely inactive as a cofactor for fatty acid biosynthesis, its presence in the chloroplasts apparently did not affect lipid biosynthesis. The pDU800-transformed plants expressing apoACP-I appeared normal. Studies using mutant spinach ACPs that are analogs of the apo form have shown that they do not significantly inhibit fatty acid synthesis *in vitro* (Jaworski, Post-Beittenmiller, and Ohlrogge, 1989). The studies reported here indicate that there was no significant inhibition by apoACP-I *in vivo*.

The observation that plants do not normally contain apoACP implies that holoACP synthase activity is sufficient to keep the pool of ACP fully phosphopantethenylated. At present we do not understand the reason for accumulation of spinach apoACP-I, but several possibilities can be considered. As discussed above, there is no evidence that apoACP-I arises from conversion of holoACP-I via a holoACP hydrolase activity; therefore, it appears to be the result of an imbalance in the relative rates of transport and prosthetic group attachment. There are at least three ways in which such an imbalance between these rates could occur. First, holoACP synthase activity may be insufficient to phosphopantethenylate the excess ACP produced in pDU800-transformed plants. However, this would be expected to affect both the tobacco and spinach ACPs, whereas only spinach apoACP-I was detected. Second, the chimeric *rbcS*:apoACP-I may be a poor substrate for the tobacco holoACP synthase, resulting in low rates of 4'-phosphopantetheine attachment specifically to the spinach apoACP-I. Third, transport directed by the *rbcS* transit peptide may be so rapid that phosphopantethenylation cannot always occur. To distinguish between these possibilities, additional ACP constructs are presently being prepared for plant transformations.

The ability to overproduce holoACP as demonstrated in this study may provide insights into the organization of plant FAS proteins and the regulation of ACP genes. FAS has evolved in yeast, animals, and some bacteria so that the individual enzyme activities are contained on one or two large multifunctional polypeptides (Wakil, Stoops, and Joshi, 1983). This may allow the stoichiometric relationships of the enzymatic activities to be more readily maintained and intermediates of FAS to be channelled more efficiently. Although the component enzymes of plant FAS are readily separable *in vitro*, their molecular organization *in vivo* is not known. There is speculation that the individual polypeptides of plant FAS may exist *in vivo* as a supramolecular structure similar to that proposed for other pathways such as the citric acid cycle (Srere, Sumegi, and Sherry, 1987) or the Calvin cycle (Gontero, Cardenas, and

Ricard, 1988). Short-chain intermediates of fatty acid synthesis are not detected, suggesting metabolic channelling. In addition, pre-incubation of FAS with ACP in some cases appears to stimulate rates of FAS in a way suggesting ACP involvement in FAS protein organization (Rutkoski and Jaworski, 1978). If the tobacco FAS enzymes exist in an ordered complex, the levels of the individual components may be coordinately regulated to maintain the proper stoichiometric relationships.

Although changes in the levels of ACP and its mRNA have been found to correlate with changes in the rates of FAS (Ohlrogge and Kuo, 1984a; Slabas et al., 1987; Han-napel and Ohlrogge, 1988), it is not known how ACP levels are regulated. By expressing the spinach ACP under control of the relatively strong *rbcS* promoter, we were able to test whether or not the steady-state level of total ACPs could be increased. It was possible that either excess ACP would be degraded, as has been demonstrated for excess rubisco small subunit when it is not associated with large subunit (Schmidt and Mishkind, 1983), or that excess ACP could interfere with the assembly of an FAS-ordered complex due to nonstoichiometric ratios of the individual components. Furthermore, if the levels of ACP could be raised, would the increase cause a compensatory decrease in the steady-state levels of the endogenous tobacco ACPs or would the increased ACP level cause alterations of lipid metabolism?

We first demonstrated that the levels of spinach holoACP-I in tobacco chloroplasts could be raised twofold to threefold above the endogenous tobacco ACP, increasing the level of total acyl carrier proteins threefold to fourfold. Second, using both protein immunoblots and *in vitro* acylation of ACP, we could detect no decrease in the levels of endogenous tobacco holoACPs in response to the increased overall ACP level. These data together indicate that, if the levels of ACP are directly regulated, the additional spinach ACP-I does not perturb this regulation sufficiently to cause a compensatory decrease in the tobacco ACP levels. They also suggest that if a supramolecular organization of plant FAS exists, it is not affected by an altered stoichiometric relationship of its individual components and it is flexible enough to allow the substitution of spinach ACP for tobacco ACP. Finally, our results indicate that approximately 5% to 20% of the spinach ACP-I expressed in tobacco leaves was in the C8-C18 acyl form (similar to levels detected in spinach), providing a clear demonstration that spinach ACP-I participated in tobacco fatty acid metabolism. Although we cannot rule out that the acyl-ACP arises from some unknown process, the only known mechanism in plants by which C8-C18 chain acyl groups are attached to ACP is through activity of the  $\beta$ -ketoacyl-ACP synthases. Therefore, the simplest interpretation of these data is that the spinach ACP was participating in the complete tobacco fatty acid synthesis cycle of reactions leading to formation of C8-C18 spinach acyl-ACP-I.



It is likely that FAS is tightly regulated *in vivo* to coordinate production of fatty acids with their utilization for glycerolipid synthesis. However, the nature of controls on plant lipid biosynthesis is not known. Given the multiple roles that ACP plays in fatty acid and glycerolipid biosynthesis, changes in ACP levels might be expected to affect lipid metabolism. In developing seeds, increases in the rates of fatty acid synthesis are temporally correlated with increases in ACP expression (Ohlrogge and Kuo, 1984a; Slabas et al., 1987; Hannapel and Ohlrogge, 1988). Kinetic data suggest that *in vivo* ACP concentrations ( $\sim 8 \mu\text{M}$ ) (Ohlrogge, Kuhn, and Stumpf, 1979) may not be saturating for some ACP-utilizing enzymes such as FAS (ACP  $K_m = 5 \mu\text{M}$ ) and malonyl-CoA:ACP transacylase (ACP  $K_m = 34 \mu\text{M}$ ). In addition, increasing the level of ACP to  $11 \mu\text{M}$  to  $55 \mu\text{M}$  *in vitro* resulted in the production of short-chain (C8 to C14) fatty acids (Huang and Stumpf, 1971; Slabas, Roberts, and Ormesher, 1982; Singh, Nee, and Pollard, 1984). It has also been suggested that ACP isoforms influence partitioning between plastidial and nonplastidial pathways of glycerolipid synthesis (Guerra, Ohlrogge, and Frentzen, 1986). However, in pDU800-transformed plants, despite the threefold to fourfold increase in holoACP levels and the participation of spinach ACP-I in lipid metabolism, there were no detectable alterations in the lipid class composition, the overall fatty acid composition, or the lipid to dry weight ratio. The results of this study therefore suggest that there are sufficient controls on plant FAS and fatty acid chain length *in vivo* to compensate for any influence of the increased ACP levels.

## METHODS

### Molecular Cloning Techniques and Construction of pDU800

All molecular biological techniques were performed according to standard protocols (Maniatis, Fritsch, and Sambrook, 1982) or manufacturers' recommendations. All enzymes were purchased from New England Biolabs, International Biotechnologies, Inc., or Boehringer Mannheim Biochemicals.

Plasmid pPB269 (Beremand et al., 1987) was digested with *Nco*I, and the single-strand ends were filled in using Klenow polymerase. The plasmid was then recircularized, generating an *Nsi*I site. The resulting plasmid (pNs269) was digested with *Nsi*I and *Eco*RI, and an *Nsi*I/*Eco*RI fragment from p2312-SH containing the *rbcS* promoter and transit peptide (Mazur and Chui, 1985) was inserted, as shown in Figure 1. To confirm that the fusion was correct, the resulting plasmid, p1490, was sequenced by the dideoxy method. Plasmid p1490 was digested with *Hind*III and *Xho*I, and the fragment containing the *rbcS* promoter, transit peptide, and mature spinach ACP sequence was ligated into *Hind*III/*Xho*I-digested pNs839, which contains the polyadenylation site from the nopaline synthase gene (Bevan, Barnes, and Chilton, 1983). The resulting plasmid, p1750, was digested with *Hind*III and *Eco*RI, and the chimeric gene was ligated into the polylinker

region of pBin19 (Bevan, 1984) to generate pDU800, in which the recombinant ACP gene was positioned between the T-DNA borders. pDU800 was introduced into *Agrobacterium tumefaciens* strain LBA4404 (Hoekema et al., 1983) by direct transformation (An et al., 1988). The transformed *Agrobacterium* was used to infect tobacco leaf discs (Rogers, Horsch, and Fraley, 1986).

### Protein Gels and Immunoblot Analyses

Leaf samples were homogenized in 5 volumes of phosphate-buffered saline (45 mM  $\text{KH}_2\text{PO}_4$ , pH 7.0, 150 mM NaCl) containing 100 mM DTT using a Teflon pestle in a microcentrifuge tube. Samples were centrifuged at 15,000 *g* for 5 min, and aliquots of the supernatant were mixed with sample buffer and loaded on either SDS or native acrylamide gel systems. SDS (15% acrylamide) and native (13% acrylamide) gel electrophoresis were according to published procedures of Laemmli (1970) and Rock and Cronan (1981), respectively. Gels were electroblotted to nitrocellulose using a continuous buffer system (39 mM glycine, 48 mM Tris, 20% (v/v) methanol). The blots were soaked in 5% formaldehyde for 5 min to 10 min, and then rinsed two to three times with water. The nitrocellulose was then processed for protein immunoblot analyses (Harlow and Lane, 1988). The primary antibody was diluted 1:250 in Blotto (5% nonfat dried milk in 10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.5% Tween 20). In some cases, relative band intensities were assessed using an LKB UltroScan densitometer.

### Assays for ApoACPs and HoloACPs

HoloACP synthase was used to convert apoACP to holoACP as previously described (Elhussein, Miernyk, and Ohlrogge, 1988) except that, in the first stage of the assay, the enzyme preparation was used directly without desalting. Assays were linear with substrate concentration over the ranges used in this study. The apoACP was extracted from a native gel in 10 mL of 10 mM Mes, pH 6.1. Polyacrylamide particles were removed from the extract, and the protein was concentrated on a 0.5-mL DE52 (Whatman) column equilibrated with 10 mM Mes, pH 6.1. The apoACP was eluted with 0.5 M LiCl, 10 mM Mes, pH 6.1. The protein was precipitated with 2% TCA to remove salt, dried, and resuspended in the holoACP synthase assay buffer.

*E. coli* acyl-ACP synthetase was used to quantitatively label tobacco and spinach ACP with  $^3\text{H}$ -palmitic acid. ACPs were extracted from 5 g of tobacco leaf by homogenizing in 25 mL of buffer (0.1 M potassium phosphate, pH 7.5, 0.015 M  $\beta$ -mercaptoethanol, 2% Triton X-100) with a Polytron (PTA 10TS probe). The homogenate was filtered through three layers of cheesecloth and then one layer of Miracloth. The filtered extract was centrifuged at 48,000*g* for 15 min. An equal volume of 0.01 M Mes, pH 6.1, was added to the supernatant, and the sample was applied to a 0.5-mL DE52 (Whatman) column. The ACPs were eluted with 0.5 M LiCl and collected in 250- $\mu\text{L}$  fractions. The fractions were heated to 65°C for 5 min and then 5- $\mu\text{L}$  and 10- $\mu\text{L}$  aliquots of each fraction were assayed for ACP using *E. coli* acyl-ACP synthetase as previously described (Kuo and Ohlrogge, 1984c). Greater than 90% of the ACP was contained in one fraction. The

peak fraction was acylated in a large-scale reaction, repurified using a DE52 column as above, and loaded onto a native gel. The gel was impregnated with EN<sup>3</sup>HANCE and exposed to Kodak X-Omat AR film. Protein concentrations were determined by the dye-binding assay of Read and Northcote (1981).

### Chloroplast Isolation

Intact chloroplasts were isolated on Percoll gradients and trypsin-treated by the method of Schreier, Reiss, and Kuntz (1988) with the following modifications. The plants were grown in the growth chamber under an 18-hr day, 6-hr dark, light regimen, and then put in the dark for 2 days. They were not re-exposed to light prior to chloroplast isolation. The tissue was homogenized with a Polytron (PTA 10TS probe) rather than a Waring blender. After treatment with trypsin or lysis followed by trypsin treatment, the samples were loaded directly onto a native gel without re-isolation on Percoll gradients.

### Plant Growth Conditions

F1 seeds were germinated on kanamycin plates to select for kan<sup>R</sup> seedlings that were used in all experiments described, except the characterization of initial transformed plants. Kan<sup>R</sup> tobacco seedlings were transferred to soil and grown in a growth chamber with a 16-hr light, 28°C, 8-hr dark, 22°C, regime. Some F1 plants were grown to the six-leaf to eight-leaf stage and then transferred to a light-tight box, and leaf samples were taken at 24-hr intervals. The samples were wrapped in foil to exclude light and stored at -70°C. After all samples were collected, the tissues were homogenized and processed for protein immunoblot analyses as above.

### Lipid Analyses

For lipid analyses, control plants transformed with vector sequences alone (pBin19) and pDU800-transformed plants were grown from F1 seeds germinated on kanamycin plates. Kan<sup>R</sup> seedlings were transferred to soil and grown in the growth chamber until leaves were harvested at the six-leaf to eight-leaf stage. Protein immunoblot analyses confirmed the presence of spinach ACP-I in the kan<sup>R</sup> pDU800 F1 plants.

Leaves were harvested and frozen in liquid nitrogen. The frozen tissue was boiled 15 min in isopropyl alcohol and homogenized in at least 10 volumes of hexane/isopropyl alcohol (3:2). Extracts were filtered, dried, and resuspended in chloroform/methanol (2:1). Individual lipid classes were isolated by thin-layer chromatography on Whatman K6 silica gel plates. Triacylglycerols and free fatty acids were separated using hexane/ether/acetic acid (80:20:1, v/v/v) as the developing solvent. Glycolipids and phospholipids were separated with chloroform/methanol/acetic acid (75:25:8, v/v/v). Standards were visualized with iodine, and the bands containing the corresponding lipids were scraped, supplemented with heptadecanoic acid (C17:0) as internal standard, and transesterified with boron trichloride/methanol (14% w/v) for 1 hr at 85°C. Reactions containing triacylglycerols were supplemented

with toluene to improve solubility. Fatty acid methyl esters were then quantitated by gas-liquid chromatography on a 15-ft × 1/8-in column packed with 15% Hi-Eff-1BP on 80/100 Gas Chrom Q2 (Alltech Associates, Deerfield, IL). For determination of total fatty acids, lyophilized tissue was directly transesterified with boron trichloride/methanol. Fatty acid methyl esters were subsequently eluted from a 0.25-g BioSil A (Bio-Rad) column with hexane/diethyl ether (95:5) and analyzed by gas chromatography.

For acetate labeling, leaf discs were submerged in 100 µL of <sup>14</sup>C-acetate (2 µCi) for 20 min and 60 min. The discs were heated to 80°C for 1 hr in boron trichloride/methanol (14% w/v) to transmethylate leaf acyl lipids. After addition of 0.9% NaCl to the reactions, fatty acid methyl esters were extracted into hexane. To facilitate detection and prevent loss of trace quantities of short-chain fatty acids, samples were supplemented with a mixture of cold C8-C18 fatty acid methyl esters. The methyl esters were subsequently fractionated according to double bond number by thin-layer chromatography on silver nitrate-impregnated silica gel (Christie, 1982). Plates were developed in hexane/ether (80:20, v/v), and spots were visualized with rhodamine 6G under long-wave ultraviolet light. Methyl esters were eluted from scraped silica with hexane/ether (2:1) and washed with 50 mM Tris HCl, pH 9.0. Individual fatty acids were then separated on Whatman KC18 plates developed in acetonitrile/methanol/water (6:3:1, v/v/v) (Christie, 1982) and analyzed by autoradiography.

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