Ectopic expression of peanut acyl carrier protein in tobacco alters fatty acid composition in the leaf and resistance to cold stress

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Abstract

Acyl carrier protein (ACP), as an essential protein cofactor, plays an important role in *de novo* synthesis of fatty acids in plastids. In this study, the expression profile of peanut (*Arachis hypogaea*) *AhACP1-1* and *AhACP1-2* was analyzed in different tissues. The expression level of *AhACP1-1* was highest in the seed, whereas expression was barely detected in the shoot, and *AhACP1-2* was expressed in every tissue analyzed with the highest expression level detected in the leaf and seed. Overexpression (OE) and antisense-inhibition (AT) of *AhACP1* in transgenic tobacco modified the transcript level of endogenous *NtACPs*, and the content of total lipids and composition of fatty acid in leaves were altered compared with the wild-type control. Transgenic OE-AhACP1 or AT-AhACP1 tobacco exhibited a significant increase or decrease in polyunsaturated C18:2 and C18:3 fatty acid content, and were more tolerant or sensitive to cold stress, respectively. It is suggested that *AhACP1* bound with C18:1 might be the specific substrate of oleoyl-ACP thioesterase or glycerol-3-phosphate acyltransferase, and participates in membrane lipid synthesis.

Additional key words: antisense, *Arabidopsis thaliana*, *Nicotiana tabacum*, overexpression, transgenic tobacco.

Introduction

In higher plants, fatty acids play a crucial role in many processes. Fatty acids are critical components of membrane lipids and cuticular waxes, in addition to being precursors to several signaling and defense compounds such as jasmonates (Somerville *et al*. 2000), and are stored in triacylglycerides as a concentrated carbon source for use during seed germination. The *de novo* synthesis of fatty acids takes place primarily in plastids (Ohlrogge *et al*. 1979). In this regard, acyl carrier protein (ACP), an essential protein cofactor carrying acyl chains of different lengths, participates in the cycles of condensation, reduction, and dehydration steps. ACP is also a cofactor for desaturation and the acyl-transfer reaction of fatty acids with different chains catalyzed by stearoyl-ACP desaturase and acyl transferase.

Several different ACP isoforms have been identified in *Arabidopsis* (Hloušek-Radojčić *et al*. 1992), spinach, soybean (Ohlrogge and Kuo 1985), rapeseed (Safford *et al*. 1988), *Cuphea lanceolata* (Voetz *et al*. 1994), and barley (Hansen and Von Wettstein-Knowles 1991), some of which are expressed constitutively and others are expressed in a tissue-specific manner (Battey and Ohlrogge 1990, Hloušek-Radojčić *et al*. 1992, Bonaventure and Ohlrogge 2002). The *Arabidopsis* genome contains five isoforms of plastidial ACP (Mekhedov *et al*. 2000). ACP1, ACP2 and ACP3 are all expressed constitutively, but ACP1 is more highly expressed in seed tissue than in the leaf or root; ACP4 is the major isoform expressed in leaves, whereas ACP5 appears to be a seed-specific isoform (Branen *et al*.

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Abbreviations: ACP - acyl carrier protein; AT - antisense mRNA; DAP - days after pegging; EST - expressed sequence tag; FAME - fatty acid methyl-ester; GC - gas chromatography; MDA - malondialdehyde; OE - overexpression; RT-PCR - reverse transcription polymerase chain reaction; SFA - saturated fatty acid; USFA - unsaturated fatty acid.
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2001). Five putative ACP cDNAs were isolated from developing peanut (*Arachis hypogaea*) seeds, of which three (*AhACP1*, *AhACP4* and *AhACP5*) were predicted to be located in chloroplasts and the others in mitochondria (Li *et al*. 2010). Why does fatty acid synthesis in plants require such diversity in ACP isoforms? One reasonable hypothesis is that different ACP isoforms have distinct patterns of tissue and developmental expression to regulate the balance between fatty acid biosynthesis for membrane lipids and that for storage lipids (Ohlrogge and Kuo 1985, Hannapel and Ohlrogge 1988). Evidence from numerous *in vitro* and *in vivo* studies indicates that ACP isoforms might be specific for some enzymes or acyl chains within the fatty acid biosynthesis pathways, and thus the fatty acid composition of oilseed or leaf lipids may be changed by altering the content and ratio of different ACP isoforms (Guerra *et al*. 1986, Schütt *et al*. 1998, Suh *et al*. 1999, Branen *et al*. 2001, 2003).

In this study, two homologous genes of peanut *AhACP1*, *AhACP1-1* and *AhACP1-2*, were characterized from a cDNA library constructed from different seed development stages (20 - 80 days after pegging; DAP) of the peanut cultivar Luhua No. 14. Changes in fatty acid composition and cold-stress tolerance in transgenic tobacco constitutively overexpressing or inhibiting *AhACP1-1* or *AhACP1-2* were analyzed.

Materials and methods

Plants, growth conditions and treatments: Seeds of peanut (*Arachis hypogaea* L. cv. Luhua No. 14) were collected at different developmental stages (20 - 80 DAP) from plants grown in the experimental field of the Shandong Academy of Agricultural Sciences and used for construction of a cDNA library. To generate plants for expression analysis, seeds were sown in a temperature-controlled room and grown at 23 ± 1 °C, 16-h photo-period and irradiance of 200 μmol m^{-2} s^{-1}. For transformation, sterile leaves from tobacco (*Nicotiana tabacum*) cultured on 1/2 Murashige and Skoog (MS) basal medium were used as explants. Transgenic (detail description see below) seedlings were screened on shoot-inducing medium (MS basic medium containing 1.0 mg dm^{-3} 6-benzylaminpurine and 0.1 mg dm^{-3} α-naphthalene acetic acid, pH 5.8) supplemented with 50 mg dm^{-3} kanamycin, and then transferred to soil and grown in a temperature-controlled room as described above. Seeds from T_1 and T_2 plants were screened on MS medium containing 50 mg dm^{-3} kanamycin. Resistant T_1 and T_2 plants were transferred to soil after 20 d and cultured in a greenhouse. All further analyses, comprising lipid composition and physiological properties, were performed using tissue from T_2 plants. For the membrane lipid peroxidation and chlorophyll fluorescence parameters, seedlings of the transgenic lines and wild-type control were transferred to an incubator, maintained at 4 °C, and leaves detached from plants treated with cold stress for 0, 2, 4, or 6 h were used for analyses.

Sequence analysis of plastidial *AhACP1-1* and *AhACP1-2* cDNA: A full-length cDNA library was constructed from RNA obtained from immature seeds using the *pBluescript II KS(+) Phagemid* kit (*Stratagene*, La Jolla, CA, USA) according to the manufacturer's instructions. In total, 16 906 expressed sequance tags (ESTs) were obtained by 5′-terminal sequencing and annotated by homologous alignment. Based on the annotation results, all ESTs homologous with plastidial ACP genes were selected and analyzed again by 5′- and 3′-terminal sequencing, and were clustered using *ContigExpress Project* software (*Invitrogen*, Carlsbad, CA, USA). Sequence analysis of the plastidial ACPs was carried out using *DNASTAR* software (*DNASTAR*, Madison, WI, USA) including the coding and amino acid sequences. Other ACP amino acid sequences from 19 plant species were downloaded from *GenBank*. The multiple sequence alignment was analyzed and a phylogenetic tree was constructed using the Neighbor-Joining method with *ClustalX v. 1.83* (Thompson *et al.* 1997) and *MEGA v. 4* (Tamura *et al*. 2007).

Expression pattern in different organs: Total RNA was isolated from seeds at different developmental stages and flowers using the CTAB method (Carra *et al*. 2007), and total RNA was extracted from the root, stem and leaf using *TRIzol*® reagent (*Invitrogen*) following the manufacturer's protocol. The first-strand cDNA was synthesized using the *PrimeScript*™ RT-PCR kit (*TaKaRa*, Shiga, Japan), using oligo (dT) as the primer and 5 μg total RNA as the template, according to the manufacturer's protocol. PCR reactions were performed using the resulting cDNA as template with the following program: 5 min at 94 °C, and 30 cycles of 30 s at 94 °C, 30 s at 55 °C, 30 s at 72 °C, and 7 min extension at 72 °C. Peanut *actin-1* was used as an internal control. Primers used for semiquantitative RT-PCR were as follows: RT-AhACP-1F (5′-TCTCTTTCTCCCTGTGAAAATG GCT-3′); RT-AhACP-1R (5′-GCCACGCACACAAAA AAAGAAGGA-3′); RT-AhACP-2F (5′-TCTCTCTCT CTGTGAAAATG-3′); RT-AhACP-2R (5′-CTTAATCT GTCTTGCTCTCA-3′); actin-1F (5′-GCAGGGCGTGAT TTAACTG-3′); and actin-1R (5′-CTCCGATCCAGA CACTGTACT-3′).

Sense and antisense constructs of *AhACP1-1* or *AhACP1-2* and tobacco transformation: The PCR products amplified from *AhACP1-1* or *AhACP1-2* cDNA, including part of the 5′-untranslated region, were cloned into the *pGEM-T-easy* vector (*Promega*, Madison, WI,

USA) using the primers ACPF (5′-TCTCTAGAACACTCTTCTCGTGCTC-3′) and ACPR (5′-CGTCTAGAAACCGCTACAAAATGGA-3′). For convenient subcloning, the *Xba*I restrictive digestion site (underlined in the preceding primer sequences) was inserted at the 5′-end of the primer. The 500 bp *Xba*I fragment was subcloned into the binary vector *pROKII* to generate the vectors *pROK-A1*(+), *pROK-A1*(−), *pROK-A2*(+) and *pROK-A2*(−). The sense or antisense construct was identified by digestion with *Hind*III. A 1260 bp fragment was generated from the sense construct and an 840 bp segment was generated from the antisense construct. Tobacco plants were transformed using the leaf disc transformation method (Horsch *et al*. 1986). Genomic DNA from leaf tissue samples of the kanamycin-resistant T_0 and T_1 plants were analyzed by PCR. The reactions were performed with 50 ng genomic DNA isolated from six-week-old greenhouse-grown transgenic tobacco plants, using *NPTII* primers and *AhACP1-1* or *AhACP1-2* gene-specific primers in a final volume of 0.02 cm^3. The gene-specific primers were the same as those used for expression analysis. The primers specific for the *NPTII* gene were 5′-TCGACGTTGTCACTGAAGCGCG-3′ (sense) and 5′-GCGGTCAGCCCATTCGCCGCC-3′ (antisense). The over-expression (OE) lines and antisense (AT) lines of these genes were designated OE-AhACP1 and AT-AhACP1, respectively. Transgene copy number was determined by segregation analyses. For segregation analysis, tobacco seeds were germinated on MS medium containing 50 mg dm^{-3} kanamycin. Homozygous T_1 lines with respect to the T-DNA loci were selected by determining the frequency of antibiotic-resistant T_2 seeds after self-pollination. Eight OE-AhACP1 and seven AT-AhACP1 homozygous lines each carrying one gene copy were obtained. Subsequently, fatty acid composition and physiological assays were performed selecting homozygous T_2 plants (OE3-12, OE5-1, AT1-2, and AT8-1) with normal phenotypes as material.

Expression analysis of transgenic tobacco: Detection of transgene expression was carried out by gene-specific RT-PCR. Leaves were harvested from four-week-old transgenic tobacco plants, grown in a greenhouse maintained at 23 ± 1 °C with a 16-h photoperiod with irradiance of 200 μmol m^{-2} s^{-1}, and immediately frozen in liquid nitrogen. Total RNA was extracted from the leaves using *TRIzol*® reagent (*Invitrogen*). First-strand cDNA was synthesized from 5 μg total RNA using the *PrimeScript* RT-PCR kit (*TaKaRa*) according to the manufacturer's instructions. PCR assays were performed with the *AhACP1* gene-specific primers described above, and with tobacco *ACTIN* gene-specific primers (YCactinF: 5′-CCCTCCCACATGCTATTCT-3′; YCactinR: 5′-AGAGCCTCCAATCCAGACA-3′) to assess the quantity and quality of the cDNA. The transcript levels of endogenous ACPs in transgenic tobacco plants were also analyzed by semiquantitative RT-PCR using the following primers: NtACP2-1 RTF: 5′-ATGGCTTCTGTTACTGGA-3′; NtACP2-2 RTF: 5′-AGGTGCATTTGAAGGGTT-3′; NtACP2 RTR: 5′-TGATTCCAAACGCTTCCT-3′; NtACP4 RTF: 5′-GTGTTCACTCAATCAGTCG-3′; and NtACP4 RTR: 5′-GCTGCTTTCTTTTCAACA-3′.

Lipid analysis: For analysis of the fatty acid composition of leaves from transgenic tobacco, at least 10 g (fresh mass) of leaves from every transgenic line at the same nodes (the sixth to eighth leaves) were collected and dried using a freeze-drier (*VirTis 2.01*; *SP Scientific*, Warminster, PA, USA). Leaf fatty acid methyl-esters (FAMEs) were prepared following a modified one-step method (Sukhija and Palmquist 1988). First, a minimum 2 g ground dried leaves were heated at 80 °C in 4 cm^3 acetyl chloride - methanol solution (1:10 v/v) and 1 cm^3 hexane containing the mixed internal standard (each concentration: 1 mg cm^{-3}) for 2 h. The mixture was cooled to room temperature, 5 cm^3 of 7 % potassium carbonate was added and the solution was mixed uniformly. After centrifugation, 2 cm^3 of the organic phase was transferred to sample bottles for gas chromatography (GC). GC analysis was performed with a *HP-6890* (*Agilent Technologies*, Santa Clara, CA, USA) equipped with a *HP-INNOWAX* polyethylene glycol capillary column (30 m × 320 μm × 0.5 μm; *Agilent Technologies*). Each sample was analyzed three times. The analytical conditions were as follows: the initial column temperature was 180 °C, increased at 4 °C min^{-1} to 200 °C, and held for 15 min; and then raised at 10 °C min^{-1} to 230 °C and held for 10 min at the final temperature. The injector and detector temperature was 250 °C, the carrier gas flow was 300 - 400 cm^3 min^{-1}, the helium make-up gas flow rate was 45 cm^3 min^{-1}, and the split ratio was 20:1.

Membrane lipid peroxidation assay: Oxidative damage to leaf lipids was estimated from the content of total 2-thiobarbituric acid (TBA) reactive substances expressed as equivalents of malondialdehyde (MDA). The TBA content was estimated by the method of Cakmak and Horst (1991). Chopped fresh leaves (0.3 g) were ground in 5 cm^3 of 0.1 % (m/v) trichloroacetic acid (TCA) and, following centrifugation at 12 000 *g* for 5 min, a 1 cm^3 aliquot from the supernatant was added to 4 cm^3 of 0.5 % (m/v) TBA in 20 % (m/v) TCA. Samples were incubated at 90 ºC for 30 min. The reaction was stopped in an ice bath. Centrifugation was performed at 10 000 *g* for 5 min, and absorbance of the supernatant was recorded at 532 nm, 600 nm, and 450 nm with a spectrophotometer (*U-3000*; *Hitachi*, Japan). More than three replicates were analyzed for every treatment.

Leaf fluorescence measurement: Chlorophyll *a* fluorescence measurements were recorded from leaves of six-week-old T_2 seedlings and wild-type control seedlings

treated with 4 °C for 0, 2, 4, or 6 h using a portable chlorophyll fluorescence monitoring system (*FMS2*; *Hansatech Instruments*, Norfolk, UK). Fully expanded leaves at a similar developmental stage were adapted for 5 min in the dark, and then placed under a modulating measuring beam with a photon flux density of 100 μmol m^{-2} s^{-1} to determine the initial fluorescence (F_0). The leaf was then irradiated with a saturating light pulse of 1 200 μmol m^{-2} s^{-1} to measure the maximum fluorescence (F_m). The ratio of variable chlorophyll fluorescence ($F_v = F_m - F_0$) to F_m (F_v/F_m) was considered as maximum efficiency of photosystem 2. Samples of each treatment were analyzed at least three times.

Statistical analysis of data: The mean and standard deviation (SD) for the fatty acid and MDA contents and fluorescence measurements were calculated, and the difference in fatty acid contents between each treatment and the control was analyzed using paired *t*-tests at 5 and 1 % levels of significance. Differences in MDA content and F_v/F_m between each treatment and the control were analyzed using two-way analysis of variance (*ANOVA*) with *SAS v. 8.0* software (*SAS Institute*, Cary, NC, USA). Comparisons between the treatments were made with the least significant difference (LSD) test at the 5 and 1 % levels of significance.

Results

Two contigs of plastidial *AhACP*, *AhACP1-1* and *AhACP1-2*, were clustered from 15 ESTs in the peanut immature-seed cDNA library. Both contigs contained the complete open reading frame of 423 bp, 5′ untranslated region (UTR) of 72 or 70 bp, and 3′ UTR of 203 or 205 bp, respectively. The two cDNA sequences showed 96 % similarity with only three nucleotides differing in the coding regions, and the major differences were located in the 5′ UTR and 3′ UTR (Fig. 1). The 5′ UTR contained two conserved motifs, a heptanucleotide CTCCGTC box and C-T-rich sequence, which are the essential regulators controlling gene expression (Bonaventure and Ohlrogge 2002, Kim *et al*. 2005). In addition, C-T-rich sequences also existed in the 3′ UTR.

AhACP1-1 and *AhACP1-2* each encoded a polypeptide of 140 amino acids with an identical sequence, which showed 81 and 76 % identities with an unknown protein of *Medicago truncatula* and *Glycine max*, and 58 and 55 % identities with NtACP2-1 and AtACP4. Phylogenetic analysis of 29 different amino acid sequences from 19 organisms showed that, except for OsACP2, the ACPs from monocotyledons and dicotyledons comprised two distinct, large evolutionary groups. AhACP1 appeared to be more closely related to NtACP2-1 and NtACP2-2 (unpublished data derived from ESTs of *Nicotiana tabacum*) than to AtACP4 and AtACP2 (Fig. 2).

Because the *AhACP1-1* and *AhACP1-2* cDNAs showed 96 % similarity, the gene-specific primers were screened in the regions including sequence diversity and, using the plasmids containing the respective gene as templates, the specificity of the PCR assay was determined (data not shown). The expression patterns of *AhACP1-1* and *AhACP1-2* in the root, shoot, leaf, flower and seed were analyzed by semiquantitative RT-PCR, using the selected primers and optimum amplification condition. The expression level of *AhACP1-1* was highest in the seed and almost no expression was detected in the shoot, and a similarly low expression level was detected in the root, leaf and flower. *AhACP1-2* was expressed in every organ analyzed, but the expression levels were highest in the seed and leaf, moderate in the root and flower, and lowest in the shoot (Fig. 3).

To elucidate the transcription level of exotic *AhACP1* in transgenic tobacco, and whether or not the expression of exotic *AhACP1* influenced transcription of endogenous ACPs, RT-PCR was carried out using tobacco *ACTIN* as an internal control (Fig. 4). The level of mRNA transcripts of *AhACP1* and those of its antisense RNA in different transgenic lines differed. The expression of endogenous *NtACPs* was also changed, especially in line OE3-12, in which transcription of *NtACP2-1* and *NtACP2-2* was increased, whereas in line AT1-2 the transcript levels of the two genes were reduced. In addition, in line AT8-1 the transcript level of another ACP gene from tobacco, *NtACP4*, was decreased markedly.

Compared with those of the control, the contents of total lipids and the main fatty acids increased in the transgenic lines OE5-1 and OE3-12 in which *AhACP1* was overexpressed, whereas in the lines with antisense *AhACP1* (AT8-1 and AT1-2) the contents of total lipids and the main fatty acids were reduced (Table 1). In particular, except for the content of C18:3 in line AT1-2, the contents of total lipids, total unsaturated fatty acids and polyunsaturated C18:2 and C18:3 fatty acids in all transgenic lines differed significantly (Table 1). The total lipid contents in leaves of the different lines differed from the control by 15.18 % (OE5-1), 18.17 % (OE3-12), 15.69 % (AT8-1) and 9.48 % (AT1-2), respectively. However, in both OE and AT, the ratio of C18:1 to total fatty acids declined, whereas the ratio of C18:3 to total fatty acids was increased. The change in the C18:2/total fatty acids ratio differed greatly between OE-AhACP1 lines and AT-AhACP1 lines. The percentage of C18:2 to total fatty acids declined markedly in the antisense lines AT8-1 and AT1-2, and increased in the overexpression lines OE5-1 and OE3-12.

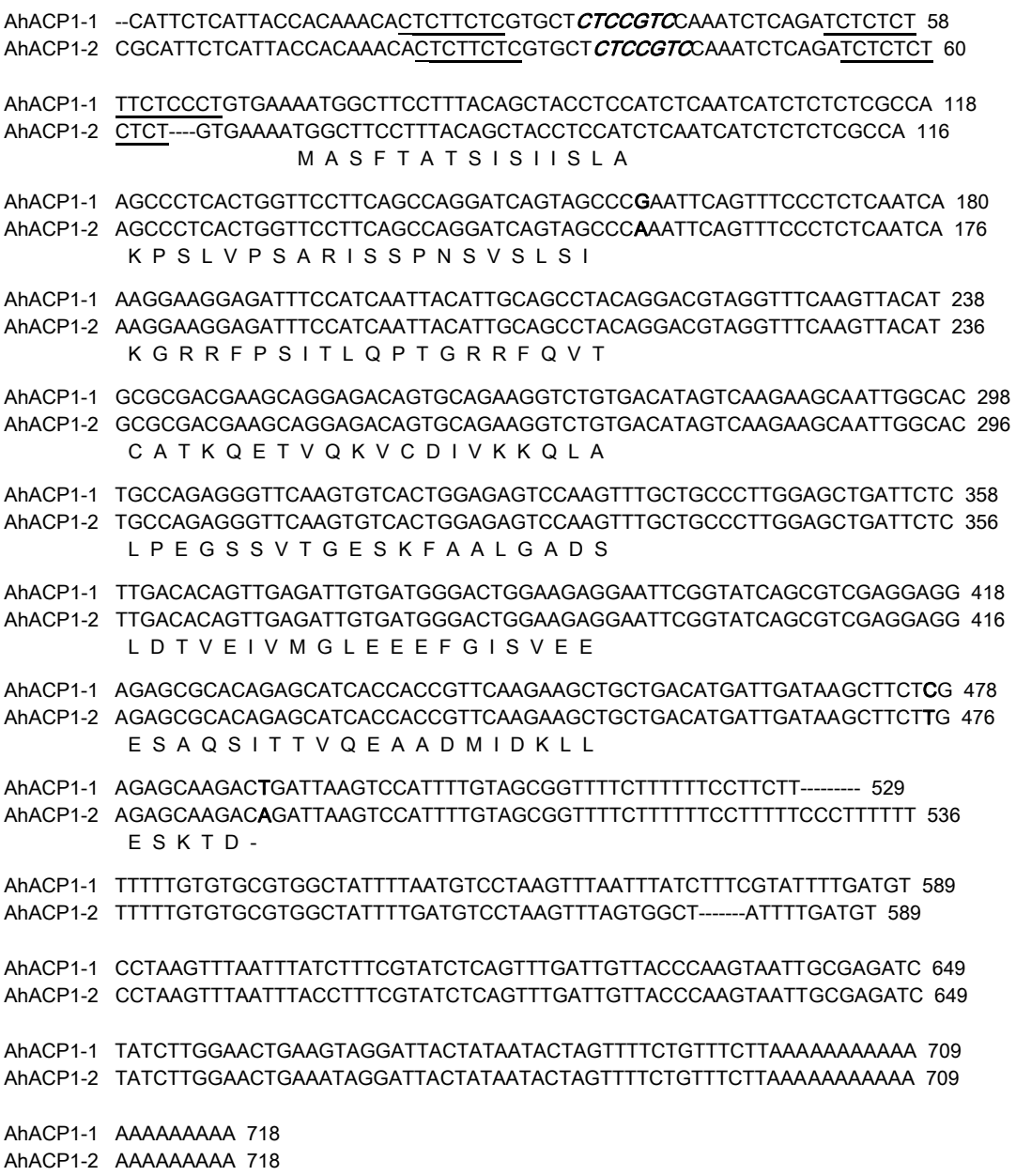

Fig. 1. Sequence alignment of the *AhACP1-1* and *AhACP1-2* contigs. Nucleotide sequences were aligned using the *CLUSTALX a*lignment algorithm. *Bold letters* indicate nucleotide differences in the open reading frame between the two sequences. *Bold italic* and *underlined letters* indicate the heptanucleotide CTCCGTC box, and CT-rich sequences in the 5′ UTR, respectively.

Because the fatty acid composition of transgenic lines changed, we hypothesized that under a low temperature (4 °C), the fluidity and antioxidative property of membrane lipids in transgenic lines might also be changed. Prior to cold treatment, except in line OE3-12, there was no obvious difference in MDA content between the transgenic lines and wild-type tobacco. The MDA content in both wild-type tobacco and the transgenic lines increased with duration of cold stress. The degree of increase was more obvious in each AT-AhACP1 line than in the control, whereas the increase was slower in all OE-AhACP1 lines (Table 2).

To determine whether the changes in fatty acid composition of sense and antisense transgenic lines affected photosynthesis, especially at low temperature, the maximum efficiency of photosystem 2 (F_v/F_m) of dark-adapted leaves from wild-type and transgenic plants, stressed at 4 °C for 0, 2, 4, or 6 h, was measured. Leaves of wild-type tobacco and the transgenic plants grown at room temperature showed similar F_v/F_m values, namely 0.766 (control), 0.763 (AT8-1), 0.794 (AT1-2), 0.757 (OE5-1) and 0.746 (OE3-12). However, with

increasing duration of cold treatment, the F_v/F_m values of all plants analyzed were reduced, especially in the AT-AhACP1 line AT8-1, compared with that of the control, and F_v/F_m values of all OE-AhACP1 lines decreased more slowly (Table 3).

Discussion

The main objective of our study was to determine whether ectopic expression of AhACP1 could change the fatty acid composition and total lipid content in receptor plants. Overexpression or antisense-inhibition of AhACP1 significantly altered the contents of total lipids and the main fatty acid composition, especially of C18:2 and C18:3 fatty acids (Table 1). Previously, several *in vivo* studies revealed that when the up- or down-regulated ACP isoform is different from the indigenous isoform present in the specific plant tissue, the fatty acid composition could be altered. For instance, over-expression of *Arabidopsis* ACP-1, which is expressed predominantly in the seed and inhibits expression of *Arabidopsis* ACP-4, the predominant ACP present in leaves, alters fatty acid composition in leaf tissue (Branen *et al*. 2001, 2003). In our study, in the transgenic tobacco line OE3-12, overexpressed *AhACP1-1* (an isoform expressed mainly in seeds) enhanced the transcript levels of endogenous *NtACP2-1* and *NtACP2-2*, whereas in the transgenic line AT1-2, in which expression of *AhACP1-1* is inhibited, the transcript levels of these two genes decreased (Fig. 4). In AT8-1, the antisense line of *AhACP1-2* (which exhibited higher transcript levels in the leaf and seed than in other organs), the transcript levels of endogenous *NtACP4* in the tobacco leaf decreased significantly and the total lipids content also decreased remarkably (Fig. 4).

ACP has a critical role in *de novo* fatty acid biosynthesis in all organisms. However, the levels of ACPs necessary to support fatty acid biosynthesis in the leaf, and the proportion of each isoform in the ACP pool, have not been determined. There is evidence that the normal pool of ACPs is sufficient to support fatty acid synthesis. Branen *et al*. (2001, 2003) indicated that the changes in leaf fatty acid composition in transgenic *Arabidopsis* with seed-predominant AtACP-1 are the result of alterations in the ACP isoform profile, and not the changes in total ACP content, and that the reduction in leaf lipid content in transgenic lines with antisense *AtACP-4* is likely owing to the decrease in total ACPs (Branen *et al*. 2001, 2003). In the present study, our results were partially consistent with this conclusion.

It is recognized that different isoforms of ACP might be responsible for specific functions in fatty acid synthesis, desaturation, acyl transfer, and other reactions. The two isoforms of ACP identified in spinach (Guerra *et al*. 1986) show different activities in the oleoyl-ACP thioesterase and oleoyl-ACP acyltransferase reactions of plastids. The isoforms Cs-ACP-I and TaACP predominantly expressed in the endosperm of *Coriandrum*

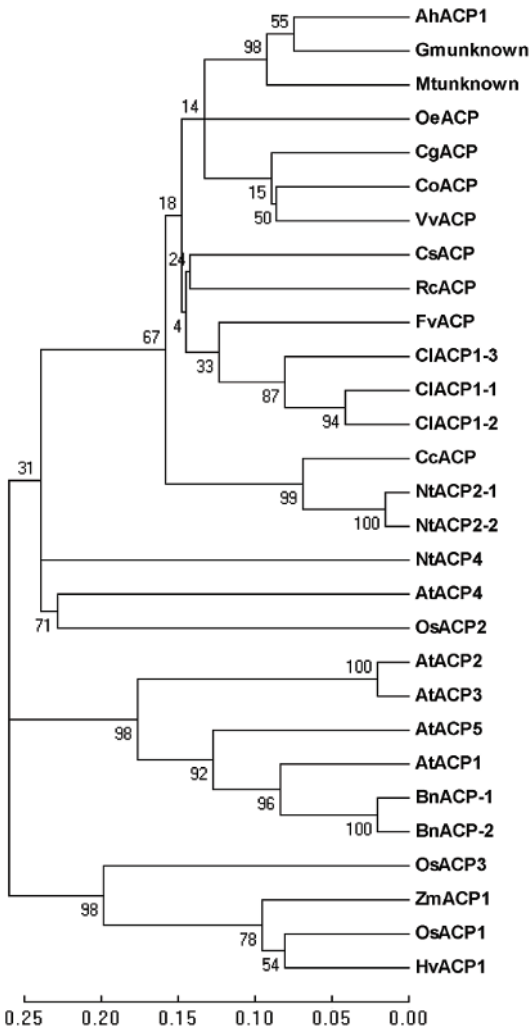

Fig. 2. Phylogenetic relationships of *Arachis hypogaea* AhACP1 with acyl carrier proteins from other angiosperms. The Neighbor-Joining tree was constructed from a data set comprising the coding sequence of *Arachis hypogaea* AhACP1 (EE127470, EE124662), the translated sequences of *Nicotiana tabacum* ESTs and other amino acid sequences from *Glycine max* (ACU14111), *Medicago truncatula* (ACJ84152), *Olea europaea* (AAL25091), *Casuarina glauca* (CAA71885), *Camellia oleifera* (ACE06753), *Vitis vinifera* (XP_002277251), *Coriandrum sativum* (AAX11453), *Ricinus communis* (EEF45474), *Fragaria vesca* (CAA04768), *Cuphea lanceolata* (CAA54714; CAA54715; CAA54716), *Capsicum chinense* (AAD21198), *Arabidopsis thaliana* (NP_187153; NP_175860; NP_564663; NP_194235; NP_198072), *Brassica napus* (CAA34248; CAA31519), *Oryza sativa* (ABA98816; ABA93958; ABF99986), *Zea mays* (ACG41313), and *Hordeum vulgare* (AAA32920).

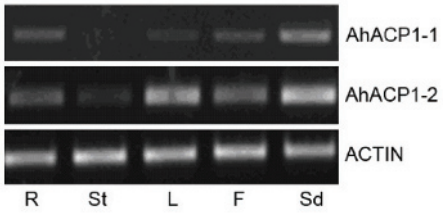

Fig. 3. Expression of *AhACP1-1* and *AhACP1-2* in different organs of peanut (R - root, St - shoot, L - leaf, F - flower, Sd - seed).

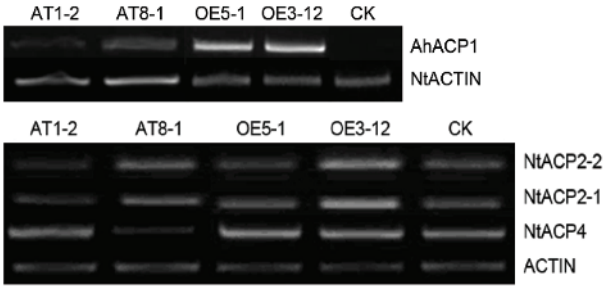

Fig. 4. Transcript levels of exotic *AhACP1* (*above*) and endogenous *NtACP* (*bellow*) in different transgenic lines and the wild-type control (CK). AT8-1 and AT1-2, lines with antisense *AhACP1*; OE5-1 and OE3-12, lines overexpressing *AhACP1*.

sativum and *Thunbergia alata* promote the production of Δ^4- hexadecenoic acid or Δ^6- hexadecenoic acid in the reaction catalyzed by acyl-ACP desaturase (Suh *et al*. 1999). Branen *et al*. (2001) suggested that stearoyl-ACP desaturase might have specificity for AtACP-1 over other ACP isoforms, and more acyl chains with 18 carbon atoms might be attached to AtACP-1. As a result, more stearic acid would be desaturated to oleic acid, and then be further desaturated to C18:2 and C18:3 (Branen *et al*. 2001). In the present study, it is suggested that *AhACP1* bound with acyl chains of C18 length might be the specific substrate of oleoyl-ACP thioesterase or glycerol-3-phosphate acyltransferase (GPAT) and, consequently, in OE-AhACP1 lines higher amounts of oleic acid than other fatty acids were transferred from the plastid to the endoplasmic reticulum (ER) and participated in fatty acid metabolism. Alternatively, more C18:1 might have occupied the sn-2 position of glycerol lipids as a result of GPAT activity and, together with C18:1 on the sn-1 position, was further dehydrogenated to produce a double-bond or triple-bond in acyl chains. Thus, the proportion of unsaturated fatty acids in membrane lipids was higher in OE-AhACP1 lines than in wild-type control and AT-AhACP1 lines.

Table 1. Leaf fatty acid contents [mg g^{-1}] in transgenic lines and the untransformed control (CK). Means ± SD, $n = 3$. The percentage of main fatty acids to total leaf fatty acids is presented in parentheses. AT8-1 and AT1-2 are lines with antisense *AhACP1*; OE5-1 and OE3-12 are lines overexpressing *AhACP1*. * and ** indicate a significant difference between the transgenic line and the control at $P < 0.05$ and $P < 0.01$, respectively. USFA - main unsaturated fatty acids; SFA - main saturated fatty acids.

Fatty acids	CK	AT8-1	AT1-2	OE5-1	OE3-12
C16:0	4.86 ± 0.04(15.3)	4.22 ± 0.11*(15.8)	4.74 ± 0.09(16.5)	5.04 ± 0.00(13.8)	5.19 ± 0.11*(13.9)
C18:0	0.69 ± 0.01(2.2)	0.67 ± 0.02(2.5)	0.61 ± 0.01(2.1)	0.74 ± 0.00(2.0)	0.80 ± 0.02(2.1)
C18:1n9	0.88 ± 0.02(2.8)	0.67 ± 0.04*(2.5)	0.64 ± 0.01*(2.2)	0.93 ± 0.00(2.5)	1.01 ± 0.05*(2.7)
C18:2	5.04 ± 0.09(15.9)	3.69 ± 0.18**(13.8)	3.42 ± 0.06**(11.9)	5.87 ± 0.01**(16.1)	6.25 ± 0.02**(16.7)
C18:3n3	19.01 ± 0.54(59.9)	16.21 ± 0.26**(60.6)	18.25 ± 0.50(63.6)	22.74 ± 0.05**(62.2)	22.89 ± 0.02**(61.1)
C20:0	0.14 ± 0.001	0.14 ± 0.003	0.14 ± 0.000	0.17 ± 0.001**	0.20 ± 0.001**
C22:0	0.08 ± 0.003	0.09 ± 0.010	0.09 ± 0.003	0.10 ± 0.000*	0.12 ± 0.005*
C24:0	0.08 ± 0.001	0.09 ± 0.002	0.10 ± 0.006*	0.09 ± 0.001	0.09 ± 0.009
C22:5	0.85 ± 0.04	0.89 ± 0.00	0.64 ± 0.01*	0.70 ± 0.01*	0.79 ± 0.07
Total	31.73 ± 0.74	26.75 ± 0.62**	28.72 ± 0.65**	36.54 ± 0.03**	37.49 ± 0.07**
USFA	25.77 ± 0.69(78.5)	21.47 ± 0.48**(76.9)	22.95 ± 0.58**(77.7)	30.24 ± 0.07**(80.8)	30.94 ± 0.17**(80.4)
SFA	5.85 ± 0.06(17.5)	5.21 ± 0.15(18.3)	5.67 ± 0.10(18.6)	6.13 ± 0.01(15.8)	6.40 ± 0.15*(16.0)

Membrane integrity and function, determined by membrane structure and fluidity, are affected by lipid composition and the degree of fatty acid desaturation in plants and other organisms (Mikami and Murata 2003). Membranes are the primary cellular structures damaged under chilling stress. Increased production of C18:3 accompanies cold acclimation in many plants (Graham and Patterson 1982), and a positive relationship exists between a higher degree of fatty acid desaturation and both cold and freezing tolerance (Steponkus *et al*. 1993, Zhang *et al*. 2010). Modifying the level of unsaturated fatty acids present in membrane lipids contributes to improved cold tolerance in cold-sensitive crops (Domínguez *et al*. 2010). In the present study, production of C18:2 and C18:3 in OE-AhACP1 lines was higher than in the wild type, which, in turn, was significantly higher than in antisense lines (Table 1). It could be speculated that the content of unsaturated fatty acids at the sn-1 and sn-2 positions of phosphatidylglycerol, which is a plant membrane lipid, could be improved in

OE-AhACP1 lines and their cold tolerance could be meliorated. Because most trienoic acids are present in thylakoid membranes, where the photosynthetic machinery is found, variation in their degree of unsaturation at low temperatures could play an important role in maintaining the photosynthetic capacity of the plants (Domínguez *et al*. 2010). Furthermore, OE-AhACP1 plants showed greater antioxidant activity and were able to maintain higher photosystem 2 activity than wild-type plants (Table 2 and 3). These results indicate that enhanced trafficking of unsaturated lipids from the ER to the thylakoids might occur to protect chloroplast function and prevent physiological disorders associated with exposure to freezing temperatures that can endanger plant survival.

Table 2. Malondialdehyde (MDA) content [nmol g^{-1}(f.m.)] in leaves of untransformed control (CK) and transgenic lines treated with 4 °C for 0, 2, 4, or 6 h. Means ± SD of three measurements taken from three different plants of each line. Differences between each treatment and the control were determined using two-way *ANOVA*. Comparisons between the treatments were performed with the least significant difference (LSD) test at the 5 % (*) and 1 % (**) levels of significance. AT8-1 and AT1-2 are lines with antisense *AhACP1*; OE5-1 and OE3-12 are lines overexpressing *AhACP1*.

Line	0 h	2 h	4 h	6 h
CK	43.8 ± 2.3	52.1 ± 3.3	56.4 ± 1.8	62.9 ± 2.6
AT8-1	46.4 ± 4.3	59.1 ± 0.8**	65.2 ± 2.2**	74.8 ± 2.6**
AT1-2	40.6 ± 1.8	47.2 ± 2.7*	50.1 ± 3.9*	68.7 ± 2.4*
OE5-1	43.9 ± 2.2	45.3 ± 1.9**	53.2 ± 0.4	56.6 ± 2.6**
OE3-12	39.0 ± 1.6*	43.1 ± 1.5**	48.2 ± 1.1**	51.1 ± 0.6**

Table 3. Maximum efficiency of photosystem 2 (F_v/F_m) of the untransformed control (CK) and transgenic tobacco lines in dark-adapted leaf tissue stressed with 4 °C for 0, 2, 4, or 6 h. Means ± SD of at least three measurements taken from three different plants of each line. Differences in F_v/F_m between each treatment and the control were determined using two-way *ANOVA*. Comparisons between the treatments were performed with the least significant difference (LSD) test at the 5 % (*) and 1 % (**) levels of significance. AT8-1 and AT1-2 are lines with antisense *AhACP1*; OE5-1 and OE3-12 are lines overexpressing *AhACP1*.

Line	0 h	2 h	4 h	6 h
CK	0.766±0.04	0.594±0.03	0.559±0.01	0.441±0.01
AT8-1	0.763±0.02	0.491±0.04**	0.382±0.03**	0.347±0.02**
AT1-2	0.794±0.01	0.575±0.01	0.512±0.02**	0.423±0.01
OE5-1	0.757±0.01	0.640±0.01**	0.537±0.01*	0.457±0.01
OE3-12	0.746±0.02	0.654±0.02**	0.552±0.01	0.482±0.00**

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