The antisense expression of *AhPEPC1* increases seed oil production in peanuts (*Arachis hypogaea* L.)

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**SUMMARY:** Although phosphoenolpyruvate carboxylases (PEPCs) are reported to be involved in fatty acid accumulation, nitrogen assimilation, and salt and drought stresses, knowledge regarding PEPC gene functions is still limited, particularly in peanuts (*Arachis hypogaea* L.). In this study, the antisense expression of the peanut PEPC isoform 1 (*AhPEPC1*) gene increased the lipid content by 5.7%–10.3%. This indicated that *AhPEPC1* might be related to plant lipid accumulation. The transgenic plants underwent more root elongation than the wild-type under salinity stress. Additionally, the specific down regulation of the *AhPEPC1* gene improved the salt tolerance in peanuts. This is the first report on the role of PEPC in lipid accumulation and salt tolerance in peanuts.

**KEYWORDS:** Antisense expression; Lipid content; Peanut; Phosphoenolpyruvate carboxylase; Salt tolerance

**RESUMEN:** La expresión antisentido *AhPEPC1* incrementa la producción de aceite de semillas de cacahuete (*Arachis hypogaea* L.). Aunque se ha demostrado que las carboxilasas fosfoenolpiruvato (PEPCs) están implicadas en la acumulación de ácidos grasos, en la asimilación de nitrógeno, y en el estrés salino e hídrico, el conocimiento respecto a las funciones del gen PEPC es todavía limitado, particularmente en cacahuete (*Arachis hypogaea* L.). En este estudio, la expresión antisentido de la isoforma (*AhPEPC1*) del gen PEPC 1 de cacahuete aumentó el contenido de lípidos en un 5.7%–10.3%. Esto indica que *AhPEPC1* podría estar relacionado con la acumulación de lípidos de plantas. Las plantas transgénicas experimentaron una mayor elongación de las raíces que la de tipo silvestre bajo estrés por salinidad. Además, la específica baja regulación del gen *AhPEPC1* mejoró la tolerancia a la sal en cacahuete. Este es el primer informe sobre el papel del gen PEPC en la acumulación de lípidos y la tolerancia a la sal en cacahuete.

**PALABRAS CLAVE:** Cacahuete; Contenido de lípidos; Expresión antisentido; Fosfoenolpiruvato carboxilasa; Tolerancia a la sal


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1. INTRODUCTION

The peanut, \textit{(Arachis hypogaea L.)} an important oilseed crop, is a major source of edible oil and the third most important source of vegetable protein, as well as being a dietary source of vitamin E and phytosterols (Pruthvi et al., 2014). The peanut seed is comprised of ~50% oil, of which ~80% are oleic (36%–67%) and linoleic (15%–43%) acids (Chi et al., 2011). Vegetable oils are the major source of edible lipids, accounting for more than 75% of the total lipids consumed across the world (Broun et al., 1999). It is quite valuable to understand lipid metabolism, which could allow us to increase the oil content and quality in peanut seeds by traditional or genetic engineering-based methods.

In the developing seeds, the partitioning of imported photosynthates to starch, storage lipid, and storage protein biosynthesis is a major agronomic concern (Ruuksa et al., 2002; Uhrig et al., 2008). Phosphoenolpyruvate (PEP) metabolism, via phosphoenolpyruvate carboxylase (PEPC; EC 4.1.1.31) and pyruvate kinase (PK; EC 2.7.1.40), plays a prominent role in partitioning oil seed carbohydrates toward plastidic fatty acid biosynthesis versus the mitochondrial production of ATP and carbon skeletons needed for amino acid inter-conversion in support of storage protein biosynthesis (Plaxton and Podestá, 2006; Uhrig et al., 2008). PEPC catalyzes PEP to yield oxaloacetate, which is required for protein biosynthesis; and PK catalyzes PEP to yield pyruvate, which is required for fatty acid synthesis (Ruuksa et al., 2002; Plaxton and Podestá, 2006). The relative activities of these two enzymes controls the ratio of protein to lipid in oilseeds. PEPC and PK activities are abundant in developing seeds and represent promising targets for metabolic engineering (Turner et al., 2005; Weber et al., 2005).

PEPC is widely distributed in bacteria and photosynthetic organisms, including plants, algae and cyanobacteria, but absent in animals and fungi (Lepiniec et al., 1994; Gehrig et al., 1998). PEPC is encoded by a small gene family, which can be classified into plant-type and bacterial-type PEPCs. Plant-type PEPCs are ~100–110 kDa polypeptides that contain a conserved N-terminal seryl phosphorylation domain and a distinguishing C-terminal tetrapeptide QNTG signature. Bacterial-type PEPCs are 116–118 kDa, larger than plant-type PEPCs, that contain a conserved N-terminal seryl phosphorylation domain and a distinguishing C-terminal tetrapeptide signature. Bacterial-type PEPCs lack the N-terminal phosphorylatable Ser residue and contain a prokaryotic-like (R/K) NTG tetrapeptide at their C-termini (Genndikas et al., 2007; O’Leary et al., 2009). In Arabidopsis, there are three PEPC genes, \textit{Atppc1}, \textit{Atppc2} and \textit{Atppc3}, encoding typical plant-type enzymes; and there is a PEPC gene termed \textit{Atppc4} that encodes a bacterial-type PEPC (Sánchez and Cejudo, 2003). Five PEPC genes have also been identified in soybean, \textit{GmPEPC1}, \textit{GmPEPC4}, \textit{GmPEPC7}, \textit{GmPEPC16} and \textit{GmPEPC17} (Sullivan et al., 2004). Interestingly, \textit{GmPEPC17} is much more similar to \textit{Atppc4} than to the other soybean PEPC sequences. It lacks the phosphorylation site, and encodes a bacterial-type PEPC. Recently, five PEPC genes, \textit{AhPEPC1}, \textit{AhPEPC2}, \textit{AhPEPC3}, \textit{AhPEPC4}, and \textit{AhPEPC5}, were cloned from peanuts, and their sequence and expression patterns in various peanut tissues were analyzed (Yu et al., 2010). However, knowledge regarding the \textit{AhPEPC} functions is still very limited.

Earlier reports showed that the \textit{AhPEPC1} gene was expressed more highly than other PEPC genes in peanut seeds (Yu et al., 2010). Here, we characterized a transgenic peanut with an antisense cDNA of \textit{AhPEPC1} under the control of a CaMV 35S promoter, for its tolerance to salinity stress. The specific down regulation of the \textit{AhPEPC1} gene increased the lipid content in peanuts. These findings expand our knowledge on the functions of the \textit{AhPEPC1} gene.

2. MATERIALS AND METHODS

2.1. Plant materials

Three T2 generations (L6, L11 and L21) of the peanut cv. huayu26, developed using \textit{Agrobacterium tumefaciens}-mediated genetic transformation as described by Chen et al. (2015), were used for the experiments. The transgenic lines and the wild-type plants were grown in the field together under normal conditions. For the salt treatments, mature seeds of wild-type and T2 transgenic lines were sown in tap-water containing 90 or 100 mM NaCl.

2.2. Vector construction and peanut transformation

A truncated ORF of \textit{AhPEPC1} (GenBank accession number EU391629) starting at base 1,784 was cloned into the \textit{pcCAMBIA 1301} expression vector using \textit{Agrobacterium} tumefaciens-mediated genetic transformation as described by Chen et al. (2015), were used for the experiments. The transgenic lines and the wild-type plants were grown in the field together under normal conditions. For the salt treatments, mature seeds of wild-type and T2 transgenic lines were sown in tap-water containing 90 or 100 mM NaCl.

2.3. PCR analysis of transgenic plants

Genomic DNA was isolated by the cetyltrimethylammonium bromide method using young leaf tissues of wild-type and transgenic peanut plants. The transgenic lines were confirmed by
PCR using marker gene hygromycin phosphotransferase (hpt)-specific primers. The 683-bp hpt gene fragment was amplified using the primers Hpt-aF (5’-CGAAAAGTTGACAGCGTCTC-3’) and Hpt-aR (5’-GCTCCATACAAGCCAACCAC-3’). The amplified products were analyzed by electrophoresis on 1.5% agarose gels.

2.4. Quantitative real-time PCR (qRT-PCR)

Total RNA was isolated from leaves using the RNeasy Mini Kit instructions (Tiangen, China). The cDNA of peanut was synthesized using M-MLV reverse transcriptase (Promega, USA) in a 25-μL reaction that contained 2 μg of total RNA. A qRT-PCR analysis was performed using a LightCycler 2.0 instrument system (Roche, Germany). The α-tubulin 5 gene (AhTUA5) was selected as a reference gene (Chi et al., 2012). Six pairs of gene-specific primers (Table 1) were designed after an analysis of the target gene sequences. qRT-PCR reactions were performed using the SYBR Premix Ex Taq polymerase (TaKaRa, Japan) according to the manufacturer’s instructions. Each 20 μL reaction comprised of 2 μL template, 10 μL 2× SYBR Premix, and 0.4 μL (200 nM) of each primer. The reactions were subjected to an initial denaturation step of 95°C·10 s−1, followed by 40 cycles of 95°C·5 s−1, 60°C·30 s−1 and 72°C·10 s−1. A melting curve analysis was performed at the end of the PCR run over the range 60–95°C, increasing the temperature stepwise by 0.5°C every 10 s. The baseline and quantification cycle were automatically determined using the Light Cycler Software. No template controls were included for each primer pair, and each PCR was carried out in triplicate.

2.5. Seed oil and protein extraction

Crude peanut oil was extracted from seeds by the Soxhlet extraction method (Harwood, 1984). Briefly, 1 g of sample was ground and then extracted with petroleum ether in a Soxhlet apparatus for 8 h. The petroleum ether was then volatilized in a ventilated place and the residue was measured using an electronic balance. The oil content was calculated by the difference of the weight of the sample and the residue.

Crude peanut protein was extracted from seeds by the Kjeldahl Method. 0.5 g sample was put in the flask and heated to digest at 420 °C after adding two pieces of catalyst and 15 mL sulfuric acid. The boiled liquid was then analyzed using the full-automatic azotometer (Kjeltte TM2300, FOSS, Danmark) according to the operating instructions.

2.6. Fatty acid composition

The fatty acid composition of peanut oil was determined using GC (Agilent 7890A, USA). Fatty acid methyl esters were prepared as follows. Clean seeds were crushed and 20 mg of sample were placed in the test tube. Then 1 mL benzene: petroleum ether (1:1), 1.5 mL sodium methoxide (0.5M), and a 2 mL saturated NaCl solution were added to the test tube in the proper sequence, and the supernatant was obtained by static. The initial column temperature was set as 210 °C and maintained for nine minutes, and then it was raised to 230 °C and held for 8 min. Helium was used as carrier gas at a flow rate of 10 mL/min. 1 μL of sample was injected using the split mode injection system.

2.7. Statistical analysis

The statistical analyses were performed using the mean values and standard errors (SEs) of three replicates per analysis. The significance of a treatment effect was determined by performing a one-way analysis of variance using SPSS 17.0 (Statistical Package For Social Sciences, SPSS Inc., Illinois).

3. RESULTS

3.1. Analysis of transgenic plants

To investigate the physiological functionality of AhPEPC1, an antisense cDNA (a truncated open reading frame starting at base 1,784) of AhPEPC1 under the control of a CaMV 3S promoter (AhPEPC1-AS) was transacted into peanuts. The transgenic lines (AhPEPC1-AS) of peanuts were selected by hygromycin and further confirmed by the PCR amplification of the hpt gene (Figure 1; Chen et al., 2015). The transgenic plants (AhPEPC1-AS)
were regenerated; and three independent lines (L6, L11 and L21) were obtained.

To check the effects of AhPEPC1-AS in the transgenic lines, the transcription levels of AhPEPC1, AhPEPC2, AhPEPC3, AhPEPC4 and AhPEPC5 in the leaves were analyzed by real-time PCR. Compared with the wild-type plants, the transcription of AhPEPC1 in the leaves of the transgenic lines was specifically down-regulated, while the transcription of AhPEPC2, AhPEPC3, AhPEPC4 and AhPEPC5 were not repressed (Figure 2). Thus, AhPEPC1 could be selectively down-regulated. However, in line L6, the expression levels of AhPEPC2, AhPEPC3, AhPEPC4 and AhPEPC5 were slightly increased (Figure 2). This result indicated that the suppression of AhPEPC1 possibly caused the improved transcription of the other PEPC genes. Thus, there were significant differences among the transgenic plants possibly because of the insertion position, or the copy number, of the transgene.

Phenotypically, all three of the transgenic lines (L6, L11 and L21) were similar to the wild-type plants under normal conditions, with no growth or developmental abnormalities (Table 2). This indicated that the insertion of the transgene did not disrupt any major endogenous functional gene(s).

3.2. The antisense expression of AhPEPC1 increases the oil content in peanut

To evaluate the effects of AhPEPC1 on seed oil content, mature seeds of the wild-type and transgenic peanut lines were simultaneously collected and analyzed. As shown in Figure 3a, the wild-type seeds contained 0.505±0.003 g lipid per g dry seed (n=3). Whereas the lipid contents of L6, L11 and L21 seeds were 0.534±0.007 (n=3), 0.542±0.006 (n=3) and 0.557±0.004 (n=3) g per g dry seed, respectively (Figure 3a). The lipid contents of the transgenic lines increased by 5.7%–10.3%. Compared with the wild-type plants, the transgenic lines had significant increases in their lipid contents. However, fatty acid compositions had no significant changes in the seeds of wild-type plants and transgenic lines (Figure 3c).
The antisense expression of \textit{AhPEPC1} increases seed oil production in peanuts (\textit{Arachis hypogaea} L.) • 5

At the same time, the seed protein contents of wild-type and transgenic peanut lines were also detected. As shown in Figure 3b, the wild-type seeds contained 0.235±0.003 g protein per g dry seed (\(n=3\)). And the protein contents of L6, L11 and L21 seeds were 0.217±0.003 (\(n=3\)), 0.205±0.003 (\(n=3\)) and 0.195±0.004 (\(n=3\)) g per g dry seed, respectively (Figure 3b). The protein contents of the transgenic lines decreased by 7.5%–17.0%. This result was consistent with the down-regulated transcription of the \textit{AhPEPC1} gene in the transgenic lines.

3.3. The specific down regulation of the \textit{AhPEPC1} gene improves salt tolerance in peanuts

\textit{Atppc1} is induced by salt stress in roots (Sanchez \textit{et al.}, 2006). However, in peanut leaves, \textit{AhPEPC1} transcription was dramatically down regulated from 8 to 72 h post-treatment with 200 mM salt (Figure 4). To assess the salt tolerance of the transgenic plants, the wild-type and transgenic plants were grown in tap water with or without a salt treatment. The average root lengths of the wild-type and transgenic 7-d-old seedlings were not significantly different in tap water without a salt treatment (Figure 5a). In tap water with the salt treatment, the transgenic plants had longer roots than the wild-type. The average root length of the transgenic plants was 2.8–4.5 times longer than that of the wild-type plants in tap water containing 90 mM NaCl (Figure 5b), and the average root length of the transgenic plants was 4.0–6.7 times longer than that of the wild-type plants in tap water containing 100 mM NaCl. Thus, \textit{AhPEPC1} suppression could partially relieve the inhibition of
4. DISCUSSION

PEPC is present in the seeds of many species (González et al., 1998; Masumoto et al., 2010) and may play a role in amino acid biosynthesis. Using immunolocalization, PEPC was detected in the protein bodies of developing wheat grains, and possibly contributes to storage-protein biosynthesis (González et al., 1998). A strong correlation between PEPC activity and protein concentration in the seeds of 13 soybean cultivars (Sugimoto et al., 1989) indicated a possible rate-limiting role of the enzyme in seed storage-protein accumulation. In developing Vicia faba cotyledons, PEPC may be important in the synthesis of organic acids that provide carbon skeletons for amino acid synthesis (Sabine et al., 1999). However, PEPC is deduced to be involved in fatty acid accumulation in seeds (Sebei et al., 2006). Class-2 PEPC complexes, formed by the interaction between bacterial-type and plant-type PEPCs, may be favorable to fatty acid biosynthesis (Gennidakis et al., 2007).

Here, we found that the antisense expression of AhPEPC1 increased lipid accumulation and decreased the protein content in the seeds of peanuts. However, Wang et al. (2012) showed that the inhibition of Atppc4 had no effects on the lipid accumulation or fatty acid composition of Arabidopsis seeds. One major reason may be that the transcription profiles of PEPC genes in the seeds of peanuts are different from those in Arabidopsis seeds. In addition, AhPEPC1 may play an important role in modulating the lipid accumulation in seeds.

Here, for the first time, we report a significant improvement in salinity tolerance in transgenic peanut plants (Figure 5). Additionally, we found that the specific down regulation of AhPEPC1 had no effects on root elongation under normal conditions. This indicated that the insertion of the transgene did not disrupt any major endogenous functional gene(s). However, AhPEPC1 suppression could partially relieve the inhibition of root growth caused by the salt treatment, and the effect became more apparent as the salt concentration increased. This result was consistent with the data shown in Figure 4 and indicated that the AhPEPC1 gene was related to plant salt tolerance.

PEPC has many different metabolic roles in higher plants. A better understanding of this enzyme family will enhance efforts to modify the content and composition of seed oils and to improve abiotic stress resistance in plants. The information generated from our study has improved our understanding of the AhPEPC1 function.

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