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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, (*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 (Ruuska *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 (Ruuska *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, and exhibit low (<40%) sequence identities with the latter. Compared with plant-type PEPCs, bacterial-type PEPCs lack the N-terminal phosphorylatable Ser residue and contain a prokaryotic-like (R/K) NTG tetrapeptide at their C-termini (Gennidakis *et al*., 2007; O'Leary *et al*., 2009). In *Arabidopsis*, there are three PEPC genes, *Atppc1*, *Atppc2* and *Atppc3*, encoding typical plant-type enzymes; and there is a PEPC gene termed *Atppc4* that encodes a bacterial-type PEPC (Sánchez and Cejudo, 2003). Five PEPC genes have also been identified in soybean, *GmPEPC1*, *GmPEPC4*, *GmPEPC7*, *GmPEPC16* and *GmPEPC17* (Sullivan *et al*., 2004). Interestingly, *GmPEPC17* is much more similar to *Atppc4* than to the other soybean PEPC sequences. It lacks the phosphorylation site, and encodes a bacterial-type PEPC. Recently, five PEPC genes, *AhPEPC1*, *AhPEPC2*, *AhPEPC3*, *AhPEPC4*, and *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 AhPEPC functions is still very limited.

Earlier reports showed that the *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 *AhPEPC1* under the control of a CaMV 35S promoter, for its tolerance to salinity stress. The specific down regulation of the *AhPEPC1* gene increased the lipid content in peanuts. These findings expand our knowledge on the functions of the *AhPEPC1* gene.

2. MATERIALS AND METHODS

2.1. Plant materials

Three T2 generations (L6, L11 and L21) of the peanut cv. huayu26, developed using *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 tapwater containing 90 or 100 mM NaCl.

2.2. Vector construction and peanut transformation

A truncated ORF of *AhPEPC1* (GenBank accession number EU391629) starting at base 1,784 was cloned into the pCAMBIA 1301 expression vector in antisense orientation at the *Xba* I/*Kpn* I sites. The pCAMBIA 1301 plasmid (GenBank accession number AF234297) is a binary vector with both the *hpt* and *GUS* genes in the T-DNA region and an extra set of *vir* genes. The construct was transformed into *Agrobacterium* host strain LBA4404 using the freeze-thaw method as described previously (Chen *et al*., 2015).

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′-CGAAAAGTTCGACAGCGTCTC-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 was 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. The relative quantification method (delta-delta Cp) was used to evaluate quantitative variation (Livak and Schmittgen, 2001).

TABLE 1. List of the primers used in real-time PCR

Name	Oligonucleotide sequence 5′–3′
qTUA5-F	CTGATGTCGCTGTGCTCTTGG
qTUA5-R	CTGTTGAGGTTGGTGTAGGTAGG
qPEPC1-F	GGCAAACGGTTCAAGATTGT
qPEPC1-R	CCTGTGGGGACTTCTTCAG
qPEPC2-F	CGGGGAAGCATTTAGACATG
qPEPC2-R	GCAGGAGTTTCTTGGTTTCG
qPEPC3-F	TATGGCTTGGCTTTGGGAAG
qPEPC3-R	CGCTCACCAAACAGCCATAG
qPEPC4-F	TCGCTGCTCTGAATGATAGG
qPEPC4-R	ACTTGTTGGGTTCAGCGAGA
qPEPC5-F	TTGTGCTGGTGATTAGTGGC
qPEPC5-R	GATCTTTCGGTTGTCGTCCT

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 ºC after adding two pieces of catalyst and 15 mL sulfuric acid. The boiled liquid was then analyzed using the full-automatic azotometer (Kjeltec 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 ºC and maintained for nine minutes, and then it was raised to 230 ºC 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 35S promoter (*AhPEPC1*-AS) was transected 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)

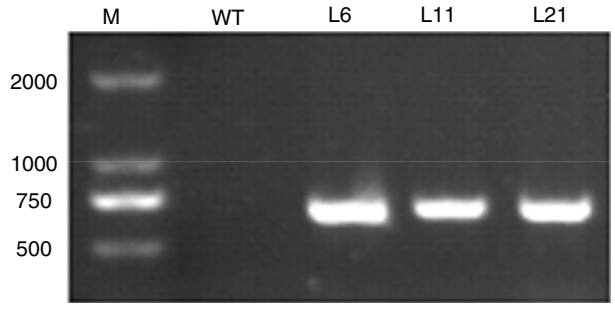

FIGURE 1. PCR detection of the *hpt* gene in transgenic peanut plants. M, molecular weight DNA markers; WT, the wild-type plant; L6, L11 and L21, the transgenic lines.

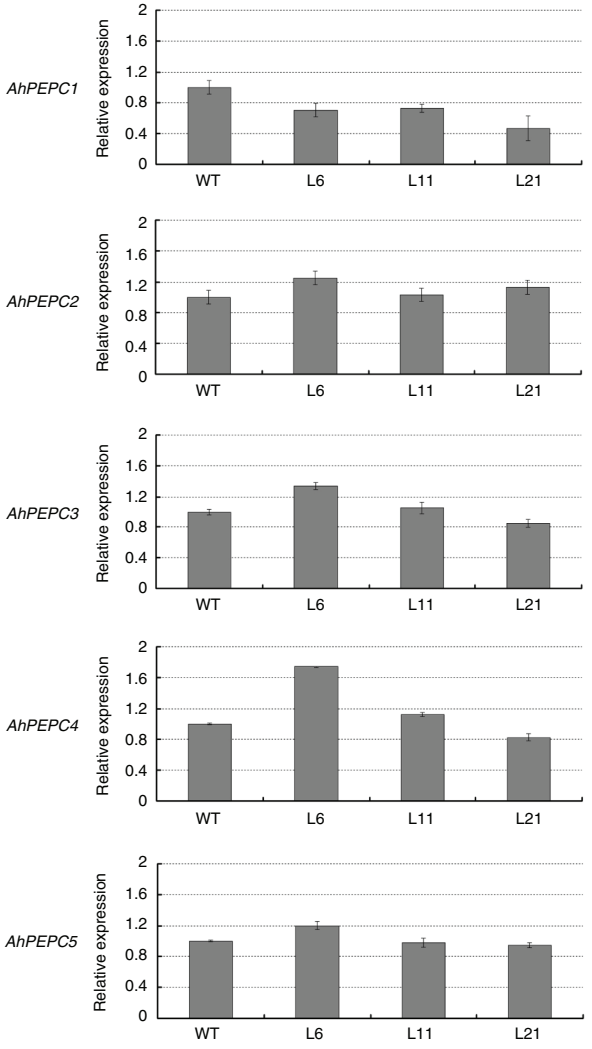

FIGURE 2. qRT-PCR analysis of peanut PEPC family members in the leaves of wild-type and transgenic plants. The relative mRNA abundance was normalized with respect to the peanut *AhTUA5* gene. The bars represent standard deviations (SDs) of three repetitions.

TABLE 2. The agronomic traits of wild-type (WT) and transgenic line

Lines	Main stem height (cm)	Branch length (cm)	Branch numbers	Pods No. per plant	Seed weight (g)
WT	33 a	40 a	7 ab	31 a	0.53 a
L6	37 b	43 b	6 a	35 a	0.55 a
L11	37 b	43 b	8 ab	40 b	0.52 a
L21	32 a	40 a	9 b	42 b	0.50 a

Note: The statistical analysis was performed using SPSS statistical software. Lowercase letters indicate significant differences at P=0.05.

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).

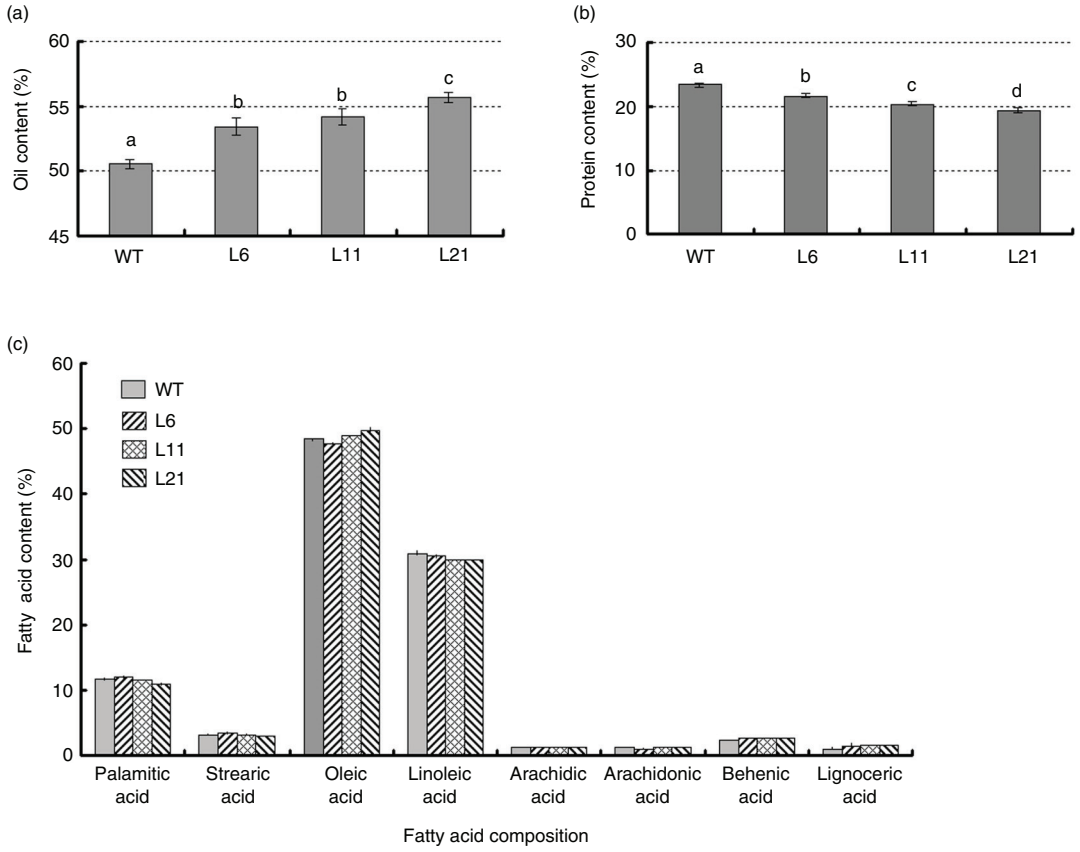

FIGURE 3. Lipid content (a), protein content (b) and fatty acid components (c) of wild-type and transgenic plants. The statistical analysis was performed using SPSS statistical software. Lowercase letters indicate significant differences at P=0.05.

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 *AhPEPC1* gene in the transgenic lines.

3.3. The specific down regulation of the *AhPEPC1* gene improves salt tolerance in peanuts

Atppc1 is induced by salt stress in roots (Sanchez *et al*., 2006). However, in peanut leaves, *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 the salt treatment (Figure 5a). In tap

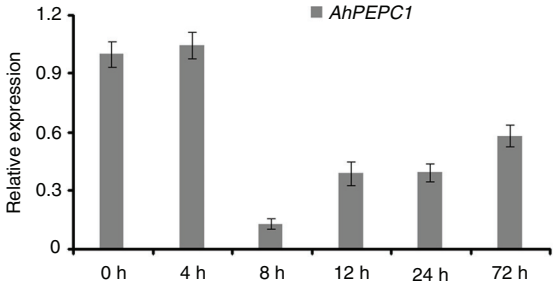

FIGURE 4. qRT-PCR analysis of the *AhPEPC1* gene in the leaves of wild-type plants treated with 200 mM salt. The relative mRNA abundance was normalized with respect to the *AhTUA5* gene. The bars represent standard deviations (SDs) of three repetitions.

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, *AhPEPC1* suppression could partially relieve the inhibition of

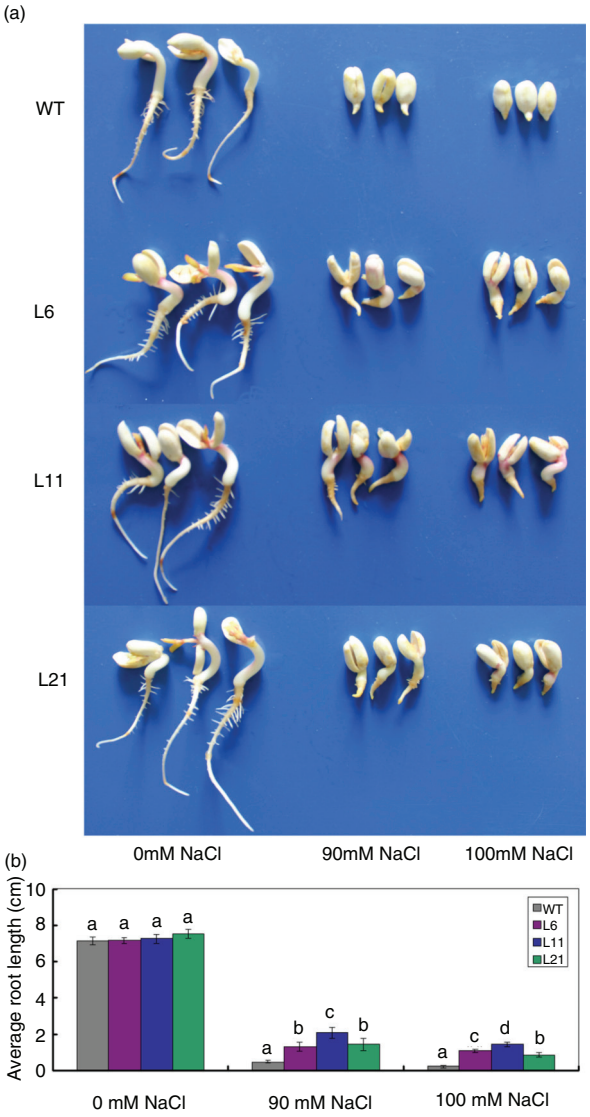

FIGURE 5. Effect of *AhPEPC1* on root elongation in tap water with or without a salt treatment. a) Wild-type and transgenic seeds sown in tap water with or without salt for 7 d. b) Root lengths of transgenic and wild-type seedlings under different treatment conditions. The statistical analysis was performed using SPSS statistical software. Lowercase letters indicate significant differences at P=0.05.

root growth caused by the salt treatment, and the effect became more apparent as the salt concentration increased.

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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REFERENCES

Broun P, Gettner S, Somerville C. 1999. Genetic engineering of plant lipids. *Annu. Rev. Nutr*. **19**, 197–216. http://dx.doi.org/10.1146/annurev.nutr.19.1.197

Chen MN, Yang QL, Wang T, Chen N, Pan LJ, Chi XY, Yang Z, Wang M, Yu SL. 2015. *Agrobacterium*-mediated genetic transformation of peanut and the efficient recovery of transgenic plants. *Can. J. Plant. Sci.* **95**, 735–744. http://dx.doi.org/10.4141/cjps-2014-012

Chi XY, Hu RB, Yang QL, Zhang XW, Pan LJ, Chen N, Chen MN, Yang Z, Wang T, He YN, Yu SL. 2012. Validation of reference genes for gene expression studies in peanut by quantitative real-time RT-PCR. *Mol. Genet. Genomics* **287**, 167–176. http://dx.doi.org/10.1007/s00438-011-0665-5

Chi XY, Yang QL, Pan LJ, Chen MN, He YN, Yang Z, Yu SL. 2011. Isolation and characterization of fatty acid desaturase genes from peanut (*Arachis hypogaea* L.). *Plant. Cell. Rep*. **30**, 1393–1404. http://dx.doi.org/10.1007/s00299-011-1048-4

Gehrig IH, Heute V, Kluge M. 1998. Toward a better knowledge of the molecular evolution of phosphoenolpyruvate carboxylase by comparison of partial cDNA sequences. *J. Mol. Evolution* **46**, 107–114. http://dx.doi.org/10.1007/pl00006277

Gennidakis S, Rao S, Greenham K, Uhrig RG, O'Leary B, Snedden WA, Lu C, Plaxton WC. 2007. Bacterial- and plant-type phosphoenolpyruvate carboxylase polypeptides interact in the heterooligomeric class-2 PEPC complex of developing castor oil seeds. *Plant. J*. **52,** 839–849. http://dx.doi.org/10.1111/j.1365-313x.2007.03274.x

González MC, Osuna L, Echevarria C, Vidal J, Cejudo FJ. 1998. Expression and localization of PEP carboxylase in developing and germinating wheat grains. *Plant. Physiol.* **116**, 1249–1258. http://dx.doi.org/10.1104/pp.116.4.1249

Harwood H. 1984. Oleochemicals as a fuel. Mechanical and economic feasibility. *J. Am. Oil Chem. Soc*. **61**, 315–324. http://dx.doi.org/10.1007/BF02678788

Lepiniec L, Vidal J, Chollet R, Gadal P, Crétin C. 1994. Phosphoenolpyruvate carboxylase: structure, regulation and evolution. *Plant. Sci.* **99**, 111–124. http://dx.doi.org/10.1016/0168-9452(94)90168-6

Livak KJ, Schmittgen TD. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* **25**, 402–408. http://dx.doi.org/10.1006/meth.2001.1262

Masumoto C, Miyazawa SI, Ohkawa H, Fukuda T, Taniguchi Y, Murayama S, Kusano M, Saito K, Fukayama H, Miyao M. 2010. Phosphoenolpyruvate carboxylase intrinsically located in the chloroplast of rice plays a crucial role in ammonium assimilation. *Proc. Natl. Acad. Sci. USA* **107**, 5226–5231. http://dx.doi.org/10.1073/pnas.0913127107

O'Leary B, Rao SK, Kim J, Plaxton WC. 2009. Bacterial-type phosphoenolpyruvate carboxylase (PEPC) functions as a catalytic and regulatory subunit of the novel class-2 PEPC complex of vascular plants. *J. Biol. Chem.* **284**, 24797–24805. http://dx.doi.org/10.1074/jbc.m109.022863

Plaxton WC, Podestá FE. 2006. The functional organization and control of plant respiration. *Crit. Rev. Plant. Sci.* **25**, 159–198. http://dx.doi.org/10.1080/07352680600563876

Pruthvi V, Narasimhan R, Nataraja KN. 2014. Simultaneous expression of abiotic stress responsive transcription factors, *AtDREB2A*, *AtHB7* and *AtABF3* improves salinity and drought tolerance in peanut (*Arachis hypogaea* L.). *Plos One* **9**, e111152. http://dx.doi.org/10.1371/journal.pone.0111152

Ruuska SA, Girke T, Benning C, Ohlrogge JB. 2002. Contrapuntal networks of gene expression during Arabidopsis seed filling. *Plant. Cell*. **14**, 1191–1206. http://dx.doi.org/10.1105/tpc.000877

Sabine G, Ute H, Christian H, Ulrich W, Hans W. 1999. Phosphoenolpyruvate carboxylase in developing seeds of *Vicia faba* L.: gene expression and metabolic regulation. *Planta* **208**, 66–72. http://dx.doi.org/10.1007/s004250050535

Sánchez R, Cejudo FJ. 2003. Identification and expression analysis of a gene encoding a bacterial-type phosphoenolpyruvate carboxylase from Arabidopsis and rice. *Plant. Physiol.* **132**, 949–957. http://dx.doi.org/10.1104/pp.102.019653

Sebei K, Ouerghi Z, Kallel H, Boukhchina S. 2006. Evolution of phosphoenolpyruvate carboxylase activity and lipid content during seed maturation of two spring rapeseed cultivars (*Brassica napus* L.). *Comptes. Rendus. Biologies* **329**, 719–725. http://dx.doi.org/10.1016/j.crvi.2006.06.002

Sugimoto T, Tanaka K, Monma M, Kawamura Y, Saio K. 1989. Phosphoenolpyruvate carboxylase level in soybean seed highly correlates to its contents of protein and lipid. *Agric. Biol. Chem*. **53**, 885–887. http://dx.doi.org/10.1271/bbb1961.53.885

Sullivan S, Jenkins GI, Nimmo HG. 2004. Roots, cycles and leaves. Expression of the phosphoenolpyruvate carboxylase kinase gene family in soybean. *Plant. Physiol.* **135**, 2078–2087. http://dx.doi.org/10.1104/pp.104.042762

Turner WL, Knowles VL, Plaxton WC. 2005. Cytosolic pyruvate kinase: subunit composition, activity, and amount in developing castor and soybean seeds, and biochemical characterization of the purified castor seed enzyme. *Planta* **222**, 1051–1062. http://dx.doi.org/10.1007/s00425-005-0044-8

Uhrig RG, O'Leary B, Spang HE, MacDonald JA, She YM, Plaxton WC. 2008. Coimmunopurification of phosphorylated bacterial- and plant-type phosphoenolpyruvate carboxylases with the plastidial pyruvate dehydrogenase complex from developing castor oil seeds. *Plant. Physiol.* **146**, 1346–1357. http://dx.doi.org/10.1104/pp.107.110361

Weber H, Borisjuk L, Wobus U. 2005. Molecular physiology of legume seed development. *Annu. Rev. Plant. Biol.* **56**, 253–279. http://dx.doi.org/10.1146/annurev.arplant.56.032604.144201

Yu SL, Pan LJ, Yang QL, Chen MN, Zhang HS. 2010. Identification and expression analysis of the phosphoenolpyruvate carboxylase gene family in peanut (*Arachis hypogaea* L.). *Agricultural Sciences in China* **9**, 477–487. http://dx.doi.org/10.1016/s1671-2927(09)60120-6