Expression Profiles of Phosphoenolpyruvate Carboxylase and Phosphoenolpyruvate Carboxylase Kinase Genes in Phalaenopsis, Implications for Regulating the Performance of Crassulacean Acid Metabolism

Chia-Yun Ping1, Fure-Chyi Chen2, Teen-Chi Cheng2, Huey-Ling Lin3, Tzong-Shyan Lin1, Wen-Ju Yang1* and Yung-I Lee4,5*

1 Department of Horticulture and Landscape Architecture, National Taiwan University, Taipei, Taiwan, 2 Department of Plant Industry, National Pingtung University of Science and Technology, Pingtung, Taiwan, 3 Department of Horticulture, National Chung Hsing University, Taichung, Taiwan, 4 Department of Biology, National Museum of Natural Science, Taichung, Taiwan, 5 Department of Life Sciences, National Chung Hsing University, Taichung, Taiwan

Phalaenopsis is one of the most important potted plants in the ornamental market of the world. Previous reports implied that crassulacean acid metabolism (CAM) orchids at their young seedling stages might perform C3 or weak CAM photosynthetic pathways, but the detailed molecular evidence is still lacking. In this study, we used a key species in white Phalaenopsis breeding line, Phalaenopsis aphrodite subsp. formosana, to study the ontogenetical changes of CAM performance in Phalaenopsis. Based on the investigations of rhythms of day/night CO2 exchange, malate contents and phosphoenolpyruvate carboxylase (PEPC) activities, it is suggested that a progressive shift from C3 to CAM occurred as the protocorms differentiated the first leaf. To understand the role of phosphoenolpyruvate carboxylase kinase (PEPC kinase) in relation to its target PEPC in CAM performance in Phalaenopsis, the expression profiles of the genes encoding PEPC (PPC) and PEPC kinase (PPCK) were measured in different developmental stages. In Phalaenopsis, two PPC isogenes were constitutively expressed over a 24-h cycle similar to the housekeeping genes in all stages, whereas the significant day/night difference in PaPPCK expression corresponds to the day/night fluctuations in PEPC activity and malate level. These results suggest that the PaPPCK gene product is most likely involved in regulation of CAM performance in different developmental stages of Phalaenopsis seedlings.

Keywords: CAM rhythm, orchid, photosynthesis transition, phosphoenolpyruvate carboxylase, phosphoenolpyruvate carboxylase kinase
INTRODUCTION

*Phalaenopsis*, an epiphytic orchid, is regarded as an obligate crassulacean acid metabolism (CAM) plant because of its succulent leaf with large and highly vacuolated mesophyll cells. Previous studies have indicated that mature plants of *Phalaenopsis* fix CO$_2$ and stored as malate inside the vacuoles at night (Endo and Ikusima, 1989; Ota et al., 1991; Cui et al., 2004; Guo and Lee, 2006). However, it has been reported that CAM orchids at their protocorm or young seedling stages might perform C$_3$ or weak CAM pathways (Goh et al., 1984). For instance, no significant day/night changes in the titratable acidity were observed during the protocorm development of *Dendrobium taurinum* (Hew and Khoo, 1980). Chen and Lee (2002) reported that in vitro young seedlings of *Phalaenopsis* absorbed more CO$_2$ during the light than in the dark. In addition, the in vitro young seedlings of *Phalaenopsis* exhibited the carbon isotopic values ($\delta^{13}$C) of $-21.4\%$ to $-19.5\%$, suggesting a weak CAM photosynthetic pathway (Lo, 2008). These findings imply that an alteration of the photosynthetic pathway from C$_3$ to CAM occurred at the early developmental stage of CAM orchids; however, definite proof has not yet been illustrated.

CAM is a water-preserving photosynthetic pathway adaptive in arid environments, in which stomata are closed during the daytime and opened at night In CAM plants, phosphoenolpyruvate carboxylase (PEPC, EC 4.1.1.31) serves as the key enzyme that fixes CO$_2$ to phosphoenolpyruvate (PEP) in the cytosol during the nighttime (Osmond and Holttum, 1981; Chollet et al., 1996; Vidal and Chollet, 1997; Nimmo, 2000; Borland et al., 2014), and produces oxaloacetic acid (OAA) stored in the vacuole through the formation of malic or citric acids. In the daytime, decarboxylation of these organic acids increases internal CO$_2$ concentration enabling CAM plants to maintain high rates of photosynthesis as the stomata are closed (Winter and Smith, 1996). This feature is an important strategy for the survival of plants in the dry habitat (Silvera et al., 2009; Rodrigues et al., 2013).

Most plants contain several PEPC isoforms encoded by a small gene family that are related to specific physiological functions (Gehrig et al., 1995; Gehrig et al., 2001). In facultative CAM plants, such as the common ice plant (*Mesembryanthemum crystallinum*) and *Kalanche* species, the CAM-specific PEPC isoform is induced to fulfill the primary carboxylation and carbon flux while switches to CAM pathway (Cushman et al., 1989; Gehrig et al., 1998). The PEPC isoforms, in addition to a CAM-specific isoform, is important for anapleurotic "housekeeping" or tissue-specific functional roles (Gehrig et al., 1998). Changes in PEPC activity are regulated by phosphorylation of a serine residue of PEPC at night that increases the catalytic activity of PEPC, and reduces the inhibitory effect on PEPC of its allosteric inhibitor, malate (Nimmo G. A. et al., 2001; Taybi et al., 2004). The phosphorylation state of PEPC is catalyzed by PEPC kinase, encoded by PPCKs (Chollet et al., 1996; Vidal and Chollet, 1997; Nimmo H. G. et al., 2001). In the common ice plant, the expression of CAM-specific PPC and PPCK are both induced during the induction of CAM (Cushman et al., 1989; Li and Chollet, 1994; Taybi et al., 2000). In *Clusia minor*, a C$_3$-CAM intermediate species, the increased expression of PEPC kinase plays an interesting role in regulating CAM performance (Borland and Griffiths, 1997). Altogether, examining the relationship between the expression of CAM-specific PPC and PPCK could be a great strategy to illustrate the developmental changes in CAM orchids.

In orchids, protocorms are tuber-shaped structures derived from germinating embryos (Arditti, 1992). The protocorm is a transitional structure, which subsequently produces the shoot and roots, resulting in the formation of a young seedling. If the protocorms or young seedlings of *Phalaenopsis* perform C$_3$ type metabolism, the developmental stage from which the plants onset the shift from C$_3$ to CAM is of interested. To elucidate the progression from C$_3$ to CAM photosynthetic pathway in *Phalaenopsis*, we investigated the expression of PPC and PPCK genes, the profiles of day/night CO$_2$ exchange, malate contents and the PEPC activity of different developmental stages. During the past decade, commercial production of *Phalaenopsis* as potted flowering plants has increased massively in the world (Griesbach, 2000; U.S. Department of Agriculture [USDA], 2017). A better understanding the regulation in CAM performance will provide insights into the micropropagation and cultivation of *Phalaenopsis*.

MATERIALS AND METHODS

Plant Materials

The mature plants of *Phalaenopsis aphrodite* subsp. *formosana* were maintained in a greenhouse with the pad-and-fan cooling system at National Museum of Natural Science, Taichung, Taiwan. To ensure good pod set and seed quantity, flowers at anthesis were hand-pollinated immediately. Mature seeds were harvested at 150 days after pollination for in vitro cultures. For in vitro seed germination, we used the 1/4-strength macro-elements (i.e., KNO$_3$, NH$_4$NO$_3$, KH$_2$PO$_4$, MgSO$_4$·7H$_2$O and CaCl$_2$·2H$_2$O) and full-strength micro-elements (i.e., FeSO$_4$·7H$_2$O, Na$_2$EDTA, MnSO$_4$·4H$_2$O, ZnSO$_4$·7H$_2$O, CuSO$_4$·5H$_2$O, KI, CoCl$_2$·6H$_2$O, H$_2$BO$_3$, NaMoO$_4$·2H$_2$O) of Murashige and Skoog (MS) medium (Murashige and Skoog, 1962), supplemented with 10 g·L$^{-1}$ sucrose (Sigma–Aldrich Co., St. Louis, MO, United States), 1 g·L$^{-1}$ peptone (Merck KGaA, Darmstadt, Germany) and 2.2 g·L$^{-1}$ Gelrite (Sigma–Aldrich Co.). The pH value of the media was adjusted to 5.7 with 1 N KOH prior to autoclaving at 121°C and 1.2 kg·cm$^{-2}$ for 15 min. When the first true leaf and root emerged, the developing protocorms were subcultured onto the culture medium containing 1/2-strength macro-elements and full-strength micro-elements of MS medium, supplemented with 10 g·L$^{-1}$ sucrose, 1 g·L$^{-1}$ peptone and 2.2 g·L$^{-1}$ Gelrite. The cultures were placed at the growth chamber at 30 µmol·m$^{-2}$·s$^{-1}$ (daylight fluorescent tube FL-20D/18, 20W, China Electric Co., Taipei) and 26 ± 2°C with a 12-h daylength (0600 to 1800 h). Different developmental stages were shown and described in Figure 1.
Measurement of Diurnal CO$_2$ Exchange

Since developing protocorms (the stages 1–3) were too small to measure the photosynthetic rate using LI-COR’s portable photosynthesis systems, we measured the changes of CO$_2$ concentrations within the culture tubes for representing their photosynthetic profiles. For obtaining the diurnal CO$_2$ change curves in vitro, one gram of protocorms were inoculated onto the culture medium in a culture tube (120 mm tall × 13 mm with 11 mm inside diameter) sealed with the aluminum foil. The cultures were placed in the growth room at 30 $\mu$mol·m$^{-2}$·s$^{-1}$ (daylight fluorescent tube FL-20D/18, 20W, China Electric Co., Taipei) and 26 ± 2°C with a 12-h daylength (0600 to 1800 h). The air within the sealed culture tube was collected by a 1-mL syringe every 2 h during a 24-h period, and the CO$_2$ concentration was determined by IR-analyzer (UNOR 610, Maihak AG, Hamburg, Germany). Each data point was measured by the accumulation of CO$_2$ concentration for 2 h within the culture tube. Before the first measurement, the aluminum foil was removed from the culture tube to balance the CO$_2$ concentration from ambient air for 5 min, then the culture tube was sealed with a rubber stopper for 2 h. After each measurement, the rubber stopper was opened to balance the CO$_2$ concentration from ambient air. Three replications were performed in each stage, and each replicate contained the measurement of three tubes. The empty tubes without protocorms were used for CO$_2$ measurements (340 ppm) for calibration.

After the leaf differentiation (stages 4, 5, and 6), gas exchange parameters were measured on the middle part of the top second leaf of a plant by a portable Infra Red Gas Analyzer (LI-COR 6400, LI-COR, Lincoln, NE, United States) with a 2 cm$^2$ leaf chamber every 2 h during a 24-h period. Photosynthetic responses were measured at leaf temperature of 30 ± 2.5°C with 350 $\mu$mol·mol$^{-1}$ CO$_2$ supplied at a flow rate of 200 $\mu$mol·s$^{-1}$. In the stages of 4 and 5, young seedlings grown in flasks (six seedlings per flask) were take out and the roots were wrapped in the moistened sphagnum moss for measurements. The leaf in the stage 4 was too small to fit a 2 cm$^2$ leaf chamber, and the lead area was estimated by LI-3100C Area Meter for correction of leaf area measurement according to the LI-6400 manual. In the stage 6, 3-year old seedlings grown in the greenhouse were measured. In these experiments, the cultures and seedlings were placed at the growth chamber at 30°C with a 12-h daylength (0600 to 1800 h). The sampled leaf was placed in the leaf chamber for 20 min before the data readings were recorded. The environmental conditions in the leaf chambers were controlled as close to those in the growth chamber as possible to reduce the variations between the sampled leaves. In this study, the completely randomized design was used, and the measurements were performed with three replications, and each replication represented the average of six seedlings of each stage.

Measurement of Malate Levels

About 0.2 g tissue from developing protocorms or leaves at each stage was sampled every 2 h during a 24-h period. In stage 5 and 6, the middle part of the top second leaf of a plant was collected for measurement. The samples were ground in a mortar with 5 mL of distilled water. The crude extracts were heated at 90°C water bath for 30 min, then cooled at room temperature. The malate content of the cooled extract was measured according to the method of Möllering and Malt (1974). The concentration of malate was determined using standard curves. For the analysis of
titratable acidity, 4 mL of the supernatant was titrated with 0.01 N NaOH solution to the end point of pH value at 8.3. The amount of NaOH was used to calculate the concentration of the titratable acid, expressed as micromoles H+ per gram fresh weight. In this study, the completely randomized design was used, and the measurements were performed with three replications, and each replication represented the average of six seedlings of each stage. The significantly different by t-test between day and night in the same stage were indicated by asterisk (*P < 0.05).

Assay of PEPC Activity
PEPC was extracted from developing protocorms and leaves of 0.1 g of each stage were sampled every 2 h during a 24-h period. In stage 5 and 6, the middle part of the top second leaf of a plant was collected for measurement. The sample was homogenized with 1.5 ml extraction buffer (50 mM Tris-HCl, pH 7.0, 10 mM MgCl₂, 1 mM EDTA, 5 mM DTT, 2.5% PVPP and 10% glycerol) according to the method of Ku et al. (1999). The homogenate was centrifuged at 17709 x g for 15 min at 4°C. PEPC activity of the supernatant was measured according to Krömer et al. (1996). Protein concentration was determined by using Bio-Rad protein assay reagent (Bradford, 1976). In this study, the completely randomized design was used, and the measurements were performed with three replications, and each replication represented the average of six seedlings of each stage. Significantly differences between day and night in the same stage, as identified by a t-test, were indicated by asterisks (*P < 0.05).

Cloning of cDNAs of PaPPCK of P. aphrodite subsp. formosana
Total RNA was extracted from the leaves of seedlings at stage 5 using RNasy Plant Mini Kit (Qiagen, Hilden, Germany) and modified as Gehrig et al. (2000). For the double-stranded cDNA synthesis, 1 μg RNA was reverse-transcribed using the SuperScript™ III kit (Invitrogen, Carlsbad, CA, United States) according to the manufacturer's instruction. The synthesized cDNA fragments and the PaPPCK degenerate primers designed from the conserved amino acid sequences in the expressed sequence tag (EST) library of Phalaenopsis (FC Chen, unpublished) were used in PCR experiments. The amplified fragment containing the partial sequence of PaPPCK that showed high sequence similarity to PPCK was identified by using a blastx algorithm in a BLAST search against the NCBI database to find the closest sequence matches in the database. Multiple alignments of amino acid sequences was performed using the ClustalW program (Thompson et al., 1994) in BioEdit Ver. 7.0.0 (Hall, 1999). Phylogenetic relationships were estimated using MEGA Ver. 4 with Neighbor-joining (NJ) analysis. The scale bar indicates a genetic distance for 0.1 amino acid substitutions per site. Every branch was supported by bootstrap analysis for 1000 replications (values of more than 70% are shown below the branches). Calcium-dependent protein kinase (CDPKs) sequences were used as the outgroup. Accession numbers of genes used for alignment and phylogenetic analyses in this study were listed in Supplementary Table S2.

RESULTS
Photosynthetic Characteristics at Different Developmental Stages
In developing protocorms, higher CO₂ concentrations within culture tubes were detected during the dark period than the light period, indicating the lack of CAM expression (Figure 2A). By stage 4, only a small amount of net CO₂ assimilation were detected during the light period, but no net CO₂ assimilation was detected during the dark period (Figure 2B). The typical net CO₂ assimilation rhythm of CAM was first observed by stage 5, which showed a major net CO₂ assimilation during the dark period.

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Malate Contents and PEPC Activities at Different Developmental Stages

From stages 0 to 2, no diurnal differences in the malate contents were observed. By stage 3, the noteworthy diurnal fluctuations in the malate contents first appeared, and the diurnal fluctuations were becoming noticeable as the seedlings becoming mature (stages 4–6) (Figure 3A). In developing protocorms and seedlings, the diurnal fluctuations in PEPC activity were similar to those in malate contents (Figure 3B).

Characterization of PEPC Isoforms in *Phalaenopsis*

The whole genome sequence dataset of *Phalaenopsis equestris* revealed two isoforms of **PPC** (i.e., PEQU07008 and PEQU14315) occurred in its genome (Cai et al., 2015), while only one copy of **PPC** (AJ300742) was found in the EST library of *P. aphrodite* subsp. *formosana* (previously known as *P. amabilis*) (Tsai et al., 2013). In this study, molecular phylogenetic analysis indicated that PEQU07008 and AJ300742 were nested in **PPC1** clade (the
FIGURE 3 | Changes of malate contents and PEPC activities in Phalaenopsis at different developmental stages. Day/night changes in (A) malate content and (B) PEPC activity in different developmental stages (stages 0–6). Malate and PEPC activity were detected in the mature seed (stage 0), the enlarged embryo (stage 1), the protocorm (stages 2 and 3) and the different developmental ages of leaf (stages 4–6). Error bars represent standard error (SE) for three independent replications with the mean of six samples.

CAM-related), while PEQU14315 was nested in PPC2 clade (the anapleurotic) (Supplementary Figure S1).

Isolation and Characterization of PaPPCK cDNA

Full-length of PaPPCK cDNA was successfully cloned from P. aphrodite subsp. formosana using a combination of RT-PCR and RACE strategies. The GenBank accession numbers of PaPPCK sequence was 2100748. The cDNA contains an open reading frame of 843 bp, flanked by 5' and 3'-untranslated sequences of 35 and 233 bp, respectively. The cDNA of PaPPCK encodes a protein of 281 amino acids that demonstrates 61∼64%, 61%, and 57% sequence identity to three rice PPCKs, ice plant PPCK, and Kalanchoe fedtschenkoi PPCK, respectively (Supplementary Figure S2). The molecular phylogenetic analysis among PaPPCK and several other PPCK orthologs revealed that PaPPCK clustered with the same group of monocots (Figure 4).

Expression and Abundance of PPC Isogenes and PaPPCK

To gain the further insight into the relationships among the CO₂ assimilation, malate content, PEPC activity and gene expression
patterns at different developing stages, the mRNA levels of PPC and PaPPCK were analyzed every 2 h over a 24 h period by quantitative real-time PCR analysis (Figure 5). As expressions of PPC isogenes in different developmental stages were measured, no obvious day/night fluctuations was observed, and their expressions were constitutive and rather constant. From stages 4 to 6, the expression pattern of PaPPCK showed a typical diurnal/nocturnal rhythm.

DISCUSSION

In Phalaenopsis, the nocturnal CO₂ uptake was not observed until stage 5 (Figure 2). During stages 1–3, no obvious depletion of CO₂ in vitro was detected, and the increase of CO₂ concentration within the culture tubes from light to dark periods may reflect the elevated respiration rate of high-energy requirements in developing protocorms (Figure 2A). Differences between day/night malate and PEPC activity was first observed at stage 3 and the breadth of day/night fluctuation enlarged as the seedlings developed (Figure 3). These results implied a progressive shift from C₃ to CAM as the protocorms differentiated the first leaf in Phalaenopsis (from stages 3–5). The age-dependent expression of CAM has been reported in a number of plants. In M. crystallinum, CAM performance is modulated ontogenetically by a genetic developmental program, although high salinity could rapidly induce CAM expression in young plants (Cushman et al., 1990; Cushman and Bohnert, 1996). Several CAM species, such as Ananas comosus, Kalanchoe fedtschenkoi, Peperomia, and Clusia species, have demonstrated that a progression from C₃ to CAM occurred as the leaves becoming matured (Jones, 1975; Holthe et al., 1987; Zotz and Winter, 1996; Vaasen et al., 2006; Winter et al., 2008). During the transitional phase from C₃ to CAM, the photosynthetic features (e.g., no significant nocturnal CO₂ uptake but having malate accumulation in the stage 4 seedlings) were similar to CAM-cycling in which plants kept their stomata closed and accumulation organic acids by recapturing the respiratory CO₂ during the night (Holthe et al., 1987; Ting et al., 1996). CAM-cycling strategy allows the maintenance of photosynthetic capacity as well as water saving during drought or in the dry season (Harris and Martin, 1991). In Phalaenopsis, upon germination, the embryo first develops into a protocorm.
before forming a plantlet. Since the differentiation of first root takes about 8 weeks after germination to uptake water efficiently, CAM-cycling may help protocorms to tolerate water deficit.

Previous reports have shown that the performance of CAM could be transcriptionally regulated at the mRNA amounts of PEPC as plants became mature or in response to stress conditions (Cushman et al., 1990; Haasg-Kerwer et al., 1992; Borland and Griffiths, 1996). To determine if the ontogenetic difference in CAM performance in *Phalaenopsis* was reflected by differences in the expression of PPC transcripts, we measured the expression and abundance of two PPC isogenes in different developmental stages. In orchids, molecular phylogenetic analysis of PPC genes indicated that PPCs can be separated into two clades that reflected their different functions, i.e., the CAM-related PPC1 and the anapleurotic function PPC2, respectively (Silvera et al., 2014; Deng et al., 2016). Our previous study (Ping et al., 2010) as well as the results in this study demonstrated that both PPC1 (the CAM-related) and PPC2 (the anapleurotic) expressed in all developmental stage regardless of the time point (Figure 5 and Supplementary Figure S3), suggesting that the day/night rhythm of PEPC activity is not likely controlled at the abundance of both PPC transcripts but another factor.

In addition to the transcriptional control of PEPC activity, the posttranslational control by PEPC kinase also plays an important role in CAM performance (Hartwell et al., 1999; Taybi et al., 2004). The activation of PEPCs in CAM is regulated through a reversible phosphorylation by PEPC kinase (Vidal and Chollet, 1997; Nimmo, 2000, 2003). PEPC kinase is encoded by a small gene family involved in a variety of functions for the precise regulation of PEPC activity (Sullivan et al., 2004; Fukayama et al., 2006; Shenton et al., 2006). In *Phalaenopsis*, so far only one gene encoding PEPC kinase has been found. In general, PaPPCK increases its expression levels as the seedlings grew up. High levels of PaPPCK transcripts were detected during the dark period and extremely low amounts during the light period, particularly in the plants of stage 3 (Figure 5B). In stage 5, the increased magnitudes of malate accumulation and PEPC activity were not as high as the level of PaPPCK expression (Figures 3, 5). The existence of a protein inhibitor of PEPC kinase may provide an additional layer of control over malate accumulation by PEPC activity (Nimmo, 2003). Indeed, the apparent day/night difference in PaPPCK expression corresponds to day/night fluctuations in PEPC activity and malate levels. In *C. minor*, a C3-CAM intermediate species, CAM performance is also achieved through the shift in *PPCK* expression (Borland and Griffiths, 1997). In facultative CAM species, e.g., *K. fedtschenkoi* and *M. crystallinum*, the phosphorylation of PEPC is primarily controlled by the abundance of the *PPCK* transcript, and hence facilitates the nocturnal CO2 uptake (Hartwell et al., 1999; Taybi et al., 2000). It is known that PPCK contains a protein kinase domain without regulatory regions indicated that its activity appears to be controlled primarily at the level of expression (Nimmo H. G. et al., 2001; Nimmo, 2003). In *Phalaenopsis*, the distinctive expression pattern of PaPPCK leads us propose that PaPPCK likely plays a critical role in regulating the phosphorylation state of PEPC during the night to CAM performance.

**CONCLUSION**

Our results demonstrated a progressive shift from C3 to CAM as the protocorms differentiated the first leaf in *Phalaenopsis* seedlings by measuring the profiles of day/night CO2 exchange, malate contents, PEPC activity and the expression of PPC isogenes and PaPPCK. Notably, in *Phalaenopsis* seedlings, the day/night fluctuations in PEPC activity and the expression of PaPPCK as well as CO2 uptake was coincident well along the developing stages. Since PPC isogenes were constitutively expressed over a 24-h cycle, PaPPCK may be involved in regulating CAM performance in *Phalaenopsis*.

**AUTHOR CONTRIBUTIONS**

Y-IL conceived the study. Y-IL, F-CC, T-SL, and W-JY designed the study. C-YP, F-CC, and T-CC performed molecular analyses. C-YP performed qPCR and biochemical experiments.
C-YP and H-LL performed photosynthesis analyses. C-YP, Y-IL, W-JY, and F-CC wrote the paper. All the authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpls.2018.01587/full#supplementary-material

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial interests that could be construed as a potential conflict of interest.

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