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A Modified Protocol for *Agrobacterium* Mediated Transformation of Tomato Plants

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Abstract

An improved protocol for *Agrobacterium*-mediated transformation was developed for tomato plant. In this study, transformation was carried out using disarmed *A. tumefaciens* strain LBA4404 harboring a binary vector pGPTV-kan (13400bp) containing the CP-gene, was used in this study. Factors that affect transformation/regeneration protocols were optimized in a series of experiments. Results indicated that exposure of cotyledonary/leaf explants to *Agrobacterium* inoculums of 0.7 OD at 600nm for 10 mins, co-cultivation in MS-liquid media, selection on kanamycin (kanamycin 50mg/ml), Augmentin, and cefotaxime (250mg/ml)-containing medium and subsequent regeneration on MS medium supplemented with 2.5mg/L BAP and 0.1 mg/L IAA resulted in transformation efficiency of 24.50%. Coat protein (CP) expression was observed in transformed tomato shoots but never in the control plants. PCR amplification of DNA extracted from the transformed tissues demonstrated the generation of the expected amplicon, corresponding to CP gene. This result strongly verifies the successful transformation of tomato plant. Moreover, this protocol may pave the way for problem solving-applications encompassing other Indian crops of economic importance.

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Introduction

Tomato (*Lycopersicum esculentum* Mill.) is an economically important crop in many countries, including India. The fruit is rich in lycopene, which has beneficial health effects. About 150 million tons of tomatoes were produced in the world in 2009. China, the largest producer, accounted for about one quarter of the global output, followed by United States and India. For one variety, plum or processing tomatoes, California accounts for 90% of U.S. production and 35% of world production. They contain the carotene lycopene, one of the most powerful natural antioxidants.

Tomato is being affected by many pest and viral disease. Besides fungal, bacterial and phytoplasmal infections, it is also affected by large number of viral diseases. Of all the diseases reported in tomato, tomato leaf curl virus

(ToLCV), a geminivirus (Geminiviridae: subgroup – III) is the most important and destructive viral pathogen in many parts of India (Pratap et al., 2011).

While the use of disease resistant cultivars may present an effective way of controlling the above diseases, genetic engineering techniques continue to play a major role in the development of disease resistant cultivars. Various factors that affect the development of techniques for the isolation and identification of many genes involved in plant disease resistance, morphology and development have been studied. In addition, different factors such as *Agrobacterium* cell density (Murray et al., 1998), regeneration and co-cultivation conditions (Hu and Phillips, 2001), addition of acetosyringone and cell competence after wounding (Murray et al., 1998) and gene constructs (Krasnyanski et al., 2001) were found to play a major role in tomato transformation. The first report of tomato transformation was forwarded by (McCormick et al., 1986) and since then, there have been numerous publications on transformation of various tomato cultivars (Saker et al., 2008). Standardization of tomato transformation procedures are, still, incomplete as different tomato cultivars vary in their response to specific treatment. The present study was undertaken to develop an efficient procedure for the production of viral resistant-transgenic tomato plants expressing coat protein (CP) gene of ToLCV.

Materials and methods

Agrobacterium-mediated tomato transformation was performed with some modifications. All culture media used in the transformation and regeneration experiments were solidified with 8g/L agar and its pH was adjusted to 5.7.

Isolation and culture of embryo from tomato seed

Embryo isolation is initiated by surface sterilization of the seed by washing in water containing few drops of tween-20 solution for 15 min with intermittent shaking under sterile conditions followed by rigorous washing of seeds with double distilled water and with 70% ethanol for 90sec and with 0.1% mercuric chloride solution for about 10 min. The seeds were then washed 3 times with double distilled water for 1, 3 and 5 min. The seeds were soaked in sterile distilled water, overnight for embryo isolation. Embryo isolation was continued in a sterile LAF by making a cut on the sharper end of the seed. Blunt side of seed was gently pressed by the blunt end of scalpel and the embryo was squeezed out. The isolated embryos were inoculated on MS basal medium and kept in a growth room that was exposed to 16 hrs light and 8 hrs dark period at 25°C. The seeds were also kept for germination without embryo isolation on MS basal medium and on wet filter paper. The plates and bottles were kept in a sterile room at 25°C.

Agrobacterium culture and OD selection

A coat protein (antiviral CP gene from ToLCV) gene cloned in *Agrobacterium tumefaciens* LBA4404 (harboring plasmid pGPTV-kan) strain was used. Optimization of *Agrobacterium* concentration for gene transfer requires its culture which was done by inoculating with 1ml of culture broth overnight grown culture of Agrobacterium LBA4404 into 11 tubes containing 10 ml of LB medium with streptomycin, kanamycin and rifampicin (10µl each of 50mg/ml concentration). All tubes were kept for incubation at 28°C on a rotary shaker and OD was taken at 600nm after each and every one hour to get a defined concentration of culture using UV spectrophotometer. Tubes having OD- 0.6, 0.7, 0.8, and 0.9 was stored at -20°C for further use. At -20°C all the metabolic activities of the bacterial cells get ceased. These cultures can be used to infect the explants (Zheng et al., 2004).

Transformation and regeneration protocol

Pretreatment is a process of preparation of explants for the agroinfection or co-cultivation. The explants should be healthy and large enough of size to sustain the transformation. Pretreatment of the explant was done by cutting the leaves of one week old embryo germinated tomato plants on the margin using a sterile scalpel to get the leaf disc of about 0.4cm×0.4cm followed by cotyledon (1cm).Old grown callus of tomato were cut into small pieces by using a sharp, sterile scalpel. All the leaf disc were placed in the regeneration medium (MS-RT, MS salts and vitamins, 30 g/L sucrose, 2.5mg/L BAP and 0.1 mg/L IAA), with axial position downward and incubated under condition of light in the growth room for 2 days at 25°C. Stem and callus were also placed in the regeneration medium (MS-RT) at 25°C for 2 days. Co-cultivation of tomato explants with Agrobacterium LBA4404 were done by using two different solutions; one with 5% sucrose solution and other with MS-basal (medium with macroelements, microelements, iron, vitamins, amino acids, and sucrose)

liquid medium. Co-cultivation of explants with *Agrobacterium* LBA4404 using 5% sucrose solution and MS-Basal medium was done by using *Agrobacterium* tumefaciens (LBA4404) culture of different OD (0.5 to 0.9) at 600nm. The culture was transferred to 2ml Eppendorf tubes and centrifuged at 6000rpm for 8 to 10 min to obtain the cell pellets and the pellets were dissolved in 10 ml 5% sucrose solution/MS-Basal liquid medium. Culture of five different OD was dissolved separately in sucrose solution/MS-Basal medium. Solution was vortexed and kept at room temperature followed by the inoculation of the pretreated explants. Pretreated explants were inoculated into the sucrose solution/MS-Basal liquid medium of *Agrobacterium* LBA4404 (harboring the plasmid pGPTV-kan with Cp gene) of different OD (0.5, 0.6, 0.7, 0.8, 0.9) After incubation the sucrose solution containing explants were swirled gently for about 10 min and were transferred to the blotting paper by using sterile forceps. Explants were dried carefully for about 10 min and were placed onto MS-RT medium (MS-RT, MS salts and vitamins, 30 g/L sucrose, 2.5mg/L BAP and 0.1 mg/L IAA). The PTC bottles were kept at 28°C in dark condition for two days.

The procedure used for the selection media transfer was standardized by using different concentrations of antibiotics and based on trial and error method. MS-RT media was melted, cooled to 45°C and 100µl of antibiotics namely augmentin, cefotaxime (250mg/ml) and kanamycin (50mg/ml) were added into the 30 ml of media. Antibiotic containing water solutions was prepared by adding 100µl of augmenting (250mg/ml) into 30ml of distilled water. The explants were washed with 0.2 % sodium hypochlorite solution for 3 min followed by washing with augmentin solution, 2 times for 5min followed by washing with double distilled water, 3 times for 5min with continuous swirling. After washing with distilled water, explants were washed with MS-liquid medium for twice and kept for drying on a sterile filter paper. When the explants are fully dried, the leaf discs, stem and callus were kept on selection medium (containing antibiotics, namely Augmentin, cefotaxime (250mg/ml) and kanamycin (50mg/ml) by using sterile forceps and kept for incubation at 26°C under light condition. Kanamycin-resistant calli obtained after a second round of selection were transferred to a fresh selection medium. Once in every two weeks, the fresh and healthy looking kanamycin-resistant calli were sub-cultured in a fresh selection medium for shoot regeneration (Tahmasebi et al., 2012).

After approximately 6–8 weeks, shoots were excised and transferred to shoot elongation medium (MS salts and vitamins, 30 g/L sucrose, 1 mg/L BAP, 0.1 mg/L IAA and 250 mg/L cefotaxime) for shoot development. The shoots obtained were transferred to rooting medium (MS salts and vitamins, 30 g/L sucrose, 1 mg/L IAA and 250 mg/L cefotaxime) for root development. A set of explants which were not co-cultivated with *Agrobacterium* were also regenerated, as described above, as a negative control. Transformation frequency was expressed as a percentage of the number of shoots recovered from kanamycin-resistant calli relative to the total number of incubated kanamycin-resistant calli. Any rooting shoot on the selection medium containing 25 mg/L kanamycin was considered as a transformant. (Sharma et al., 2009).

Effect of *Agrobacterium* culture density

Bacterial densities (OD600) ranging from 0.5 to 0.9 were tested to determine the optimum density for transformation.

Effect of Sucrose solution and MS liquid medium for co-cultivation

Sucrose solution (10%) and MS-Basal liquid medium were tested to determine the optimum medium for transformation.

Molecular verification of the transformant

To confirm the presence of the CP gene in the regenerated transformed plants, total DNAs were isolated from both transformed and non transformed (negative control) plant samples and were used as templates for PCR. Plasmid DNA was also isolated and used as a positive control.

Rapid plant DNA extraction

Rapid plant DNA extraction was carried out following CTAB (cetyl trimethyl ammonium bromide) protocol, which is a modification of the method of Doyle and Doyle (1987).

Polymerase Chain Reaction (PCR)

The presence of CP gene was investigated by PCR amplification. Specific oligonucleotide primers for CP gene were ‘forward’: 5-ATGGCGAAGCGACCAG-3

and 'reverse': 5-TTAATTTGTGACCGAATCAT-3. CP gene is of 771bp or ≈800bp. Amplified DNA fragments were electrophoresed on 1.0% agarose gel and detected by ethidium bromide staining and photographed under Gel Doc.The presence of the target band in the transformants and its absence in the untransformed plants is considered as a proof of successful transformation (Tumer et al., 1987; Fuchs et al., 1996).

Results and discussion

A protocol for transformation and regeneration of tomato plants has been developed on the basis of the results of the optimization experiments conducted in the present study.

Embryogenesis

Isolated embryo from the tomato seeds were partially grown into cotyledons after 7 days and fully grown after 15 days of incubation (Fig. 1, A). Surface sterilized seeds were grown on wet filter paper into cotyledons after one week (Fig. 1, B). Overnight water soaked seeds were neither grown on MS- Basal media nor on the wet filter paper (Fig. 1, C, D).

Fig. 1: Explant culture. (A) Regenerated cotyledons from embryo (B) Germinated sterile seeds on wet filter paper (C) Non-germinated overnight soaked seeds on MS-basal medium (D) Non-germinated overnight soaked seeds on wet filter paper.

Agrobacterium culture

Agrobacterium LBA4404 (containing plasmid pGPTV-kan with CP gene) used for desired gene transfer was grown into LB medium and specific OD (OD- 0.5, 0.6, 0.7, 0.8, 0.9,) was selected. Time of incubation and growth of cells were plotted (X and Y) that shows the lag and log phase of growth (Fig. 2).

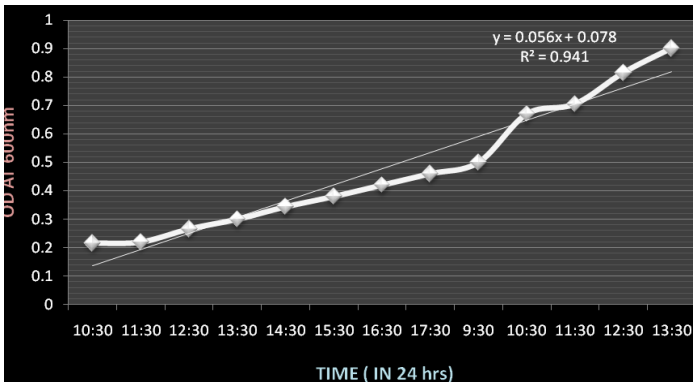

Fig. 2: Growth curve of *Agrobacterium tumefaciens*.

Pretreatment, co-cultivation and selection media transfer

Cotyledon and leaf explants of 15 days old seedlings from the embryo generated plantlet were cut and incubated on MS-RT medium for two days. Pretreatment allows the explants to get adapted on the artificial media. Fig. 3 (A) shows the pretreatment of explants.

In co-cultivation of explants, 5% sucrose with *Agrobacterium* cells and MS-liquid media with bacteria were used to infect the plant cells. At this point, the bacterial cells attach to the plant tissue for further infection. After a 10min incubation with the bacterial solution the explants were incubated on the MS-RT medium in dark for 2 days. Bacterial cells activate their vir genes for gene transfer and the gene of interest can be transferred into plant cells within these two days Fig. 3 (B) shows the co-cultivation flask. Fig. 3 (C) and (D) shows selection media transfer. Co-cultivation of

explants using MS-liquid medium showed more efficiency of transformation than that of 5% sucrose (Beachy et al., 1990; Murashige and Skoog, 1962; Dellaporta et al., 1983).

After two days of co-cultivation the explants were transferred to a selection medium containing antibiotics (Augmentin, Cefatoxime, and kanamycin). Out of 102 explants only 53 survived. Kanamycin kills the non-transformed plant cells, while transformed cells express the kanamycin resistant marker gene and stays alive. Cefatoxime kills the *Agrobacterium* cells present on the plant cells and Augmentin inhibits the growth of other microbes. Sub culturing of regenerated plantlet was done after the survival of explants on selection media (Spena et al., 1992; Pilowsky and Cohen, 1990).

Fig. 3 (E) and (F) clearly shows the difference between control plant and transformed plant. Transformed explants were grown into plantlets while control explants (without the desired gene) died because of susceptibility to kanamycin.

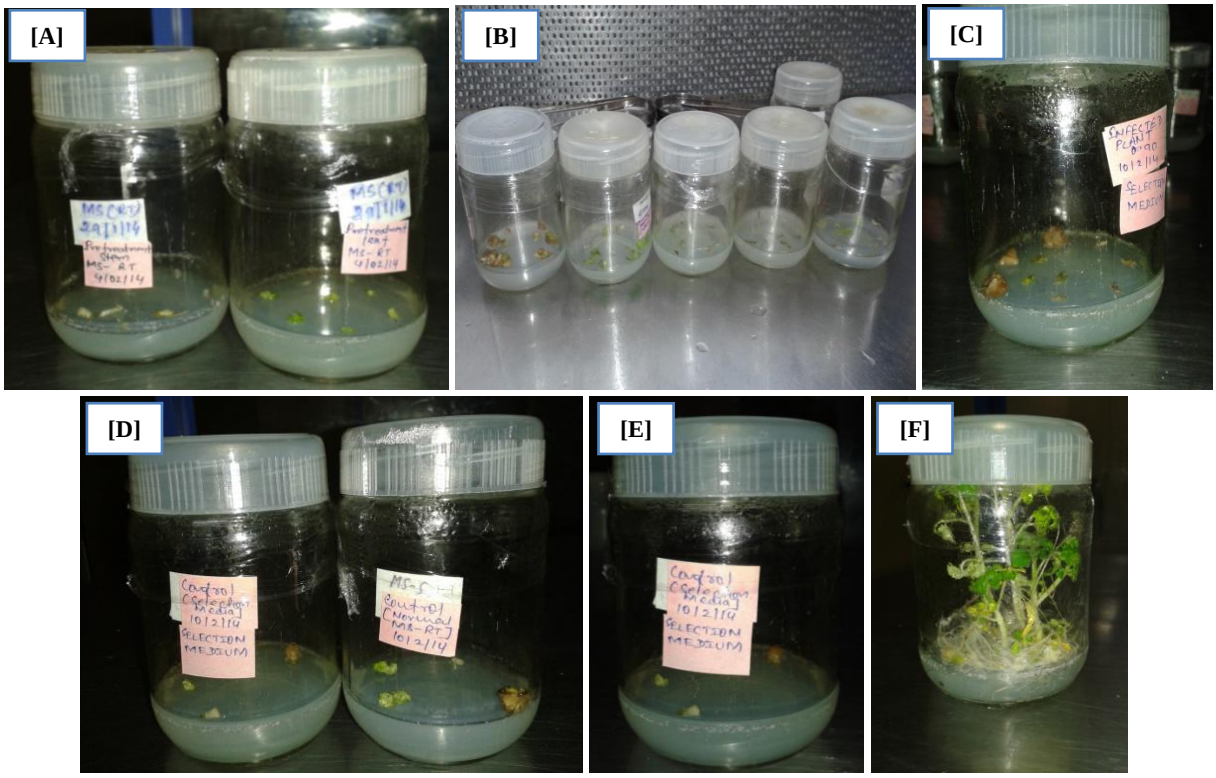

Fig. 3: Pretreatment, co-cultivation, Selection media transfer and regeneration of transgenic plant. (A) Pretreatment of explant (B) Co-cultivation of explant with different concentration of *Agrobacterium* culture (C) Transfer of co-cultivated explants on selection media (D) Transfer of control plants on selection mediaand MS-RT medium (E) Control explants on selection medium (F) Fully grown transformed tomato plantlet on selection media.[20]

The results of survivals of transformed and non transformed plantlets at various stages are summarized in Table 1. It also signifies the transformation efficiency of the experiment. The frequency of survival of explants on selection media was found to be about (51.96%, 53/102), which was less than non transformed control explants (75.0%, 75/100) (Zhuk and Rassokha, 1992).

Direct shoot initiation rather than the callus, was observed at the cut edge of the proximal end. Each explant produced a number of shoots from several trans-formation events. The true shoots were subsequently transferred on fresh selection medium for shoot elongation. About 48.03%, (49/102) trans-formed shoots survived during shoot elongation and the elongated shoots were placed in a rooting medium. Root development was carried out and the frequency of root development was 15.68%, (16/102) (Kara et al., 1994).

Table 2 shows the transformation efficiency at different OD of *Agrobacterium* culture. From the current table it is clear that maximum infectivity and transformation efficiency was achieved from 0.7 OD of culture.

Table 1. Percentage of transformation and survival of transformed plant.

Stage	Co-cultivated		Control	
	Survived/Total	Percentage	Survived/Total	Percentage
Co-cultivation	79/102	77.45	90/100	90
Growth on selection media	53/102	51.96	75/100	75
Shoot elongation	49/102	48.03	70/100	70
Rooting	16/102	15.68	65/100	65

Control plants were grown on MS-RT medium without any co-cultivation with *Agrobacterium*.

Table 2. Transformation efficiency at different OD of *Agrobacterium* culture.

Optical density of culture	No of explants transformed	Transformation percentage
0.5	5/ 102	4.90
0.6	10/102	9.80
0.7	25/102	24.50
0.8	9/ 102	8.82
0.9	4 /102	3.92

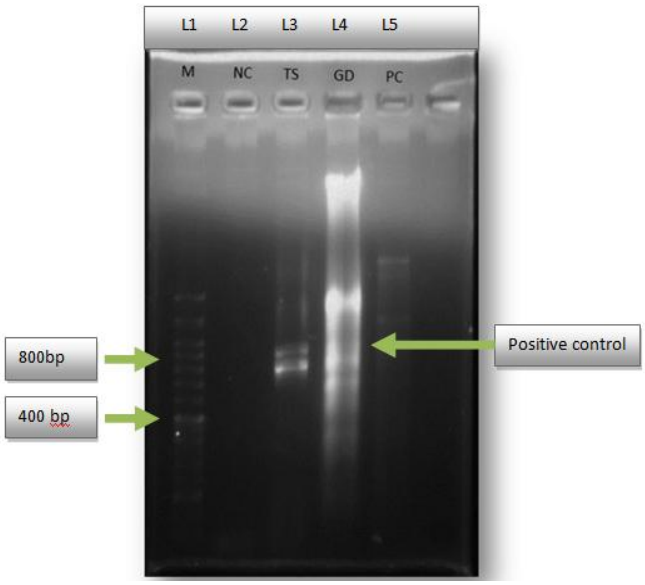

Fig. 4: Gel Electrophoresis of PCR product. Lane1 (L1): 100kb marker, Lane2 (L2): Negative control of PCR, Lane3 (L3): PCR amplified product of transformed plant (Test sample), lane4 (L4): Genomic DNA of tomato, lane 5 (L5): positive control for CP DNA.

Polymerase chain reaction and confirmation of Coat protein (CP) transfer by gel electrophoresis

Result of polymerase chain reaction followed by gel electrophoresis in Fig. 4 confirms the presence of coat protein in the tomato genome of transformed plant. In the gel electrophoresis, lane 1 contains marker DNA band of 100bp, lane 2 contains negative control for PCR i.e., genomic DNA of control tomato plant, lane 3 contains the amplified PCR product, lane 4 contains the genomic DNA of tomato, and lane 5 contains the positive control for coat protein. PCR amplified product (≈800bp) band matches with positive control (≈800bp) and shows that it is of 800bp. This confirms the amplification of coat protein sequence in the transformed plantlet.

Embryo culture and *in-vitro* seed germination is very important and major step in plant tissue culture. It provides the plant material being used for transformation experiment. Embryo was grown on MS-Basal medium and gives rise to cotyledons and leaves after 14 days as written on review articles. All overnight water soaked tomato seeds failed to germinate under in-vitro condition. This result was different from the result of review articles and shows human error and seeds potentiality to germinate *in-vitro*. All surface sterile seeds germinated on wet filter paper and shows that embryo needs only water as an external source for germination. Seed contains endosperm that supplies all the nutrients for germination and growth.

Agrobacterium LBA4404 containing the plasmid pGPTV-kan with Cp gene was cultured in LB medium. LB medium with rifampicin, streptomycin and kanamycin was used to select cells. OD selection was done for determination of highest efficiency of bacterium to transform a plant cell. Results shows that *Agrobacterium* culture of OD-0.7 at 600nm was most efficient in transformation with an efficiency of 24.50%. This result differs from the review article and may be due to different tomato variety, or different type of explants.

Pretreatment of explants was required for adaptation of explants to the media. Pretreatment was done by incubating the explants on MS-RT media for 2 days.

Co-cultivation of explants was done using suspension of cells in MS-liquid medium and in 5% sucrose solution. Co-cultivation allows the bacterial cells to transfer the desired DNA into the plant cells. Plant cell integrates the DNA into the genome randomly and expresses the foreign DNA. Co-cultivation followed by selection media transfer shown that MS-liquid suspension of bacterium is more efficient in transferring the DNA than any other method as described in the review literature.

Selection media transfer, selects the transformed cells on the basis of the presence of marker gene into the genome of transformed explants. The marker gene is kanamycin resistant gene that allows the growth of transformed plantlet onto kanamycin containing medium. A very high percentage of cell survival had been reported by standardizing the procedure of transfer of co-cultivated explants onto selection medium. Transformation efficiency was found to be higher when compared with review article and shown that the standardized protocol as a better option for transformation of tomato plant.

In order to confirm the gene transfer in our study, PCR was done using the transformed tissue DNA and CP specific primers. The Gel electrophoresis confirms the presence of CP DNA in the tomato plant. Many review articles have shown that the transformed plant are resistant to the virus under greenhouse condition but large no of field trail is required for the confirmation of resistance towards the Tomato leaf curl virus.

Summary and conclusion

Tomato leaf curl virus disease is a devastating disease of tomato varieties in India. Efforts have been made to develop disease-resistant varieties of tomato by conventional breeding, but none has been able to produce a potent virus-resistant variety against the disease. However, several researchers have attempted *Agrobacterium* mediated genetic transformation to produce transgenic tomato plants expressing the CP gene of ToLCV.

Moreover, our efforts in this direction are a preliminary process to achieve highest transformation efficiency through a modified protocol. The protocols have been developed for transferring a foreign gene into the tomato plant that has a wide scope in generation of transgenic plants using other genes of interest.

The prime area of interest after our study is detecting the position effect of transferred gene into the tomato plant. *Agrobacterium* method of transformation follows random integration of transgene into the genome. Integration into a functional gene disrupts the normal plant physiology. So considerable efforts have to be made, to eradicate the problem of position effect.

Conflict of interest statement

Authors declare that they have no conflict of interest.

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