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Demonstration of CRISPR/Cas9-mediated *PDS* gene editing and Possibility of enhancing Shelf Life of Commercially Important Tomato variety

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ABSTRACT: CRISPR/Cas9 has been a popular tool for introducing precise mutations at the target site among different crop species to improve several required traits beneficial to farmers and breeders worldwide. Tomato is a model vegetable crop belonging to the family Solanaceae. Here in present study, the *phytoene desaturase* (PDS) gene from tomato was edited using CRISPR/Cas9 technique. The *PDS* gene is involved in carotenoid biosynthesis pathway and its editing would lead to albinism in plants. The sgRNA for *PDS* gene was designed and introduced into tomato system through leaf-disc method leading to precise mutation at target loci. The editing at desired loci was detected in 20 % of the T_0 transgenic tomato plants. Rigorous screening and confirmation are always necessary for detecting true CRISPR edits. For screening of the *PDS* gene editing, first the T-DNA integration was confirmed through PCR. These plants were further analysed for mutation detection and analysis through Cel-1 assay and Sanger sequencing. About 2 bp deletion was observed in mutated plants at 3-4 bp upstream of PAM site at target loci. The editing of *PDS* confirmed that the technique can be successfully applied further for editing of *Pectate lyase* gene in commercially important tomato varieties for enhancing shelf life, which is a complex trait governed by a number of different genes and thus, a real challenge.

Keywords: Phytoene desaturase, tomato, CRISPR/Cas9, Shelf life, Pectate lyase.

INTRODUCTION

Tomato (*Solanum lycopersicum* L.) belongs to Solanaceae family ordinarily called nightshades, is one of the most consumed vegetable crops worldwide. The Solanaceae family has 100 genera and more than 3000 species including potato, tobacco, chilli and eggplant (Knapp 2002). Among these Solanaceae family members, it was hypothesized that tomato was domesticated either in Peru or Mexico. But later on, it was assumed that Mexico is the probable site of domestication and Peru is the centre of diversity of tomato. Tomato is considered as a model plant species, with diploid chromosome number of 24. Also, tomato is deemed as protective food as it is a rich source of minerals, antioxidants, carotenoids (especially lycopene), vitamins (A, B and C) and organic acids (Khachik *et al.*, 2002).

Genome editing at precise positions offers great advantages in research and crop improvement by generating tailored modifications at target genome regions. Genome editing tools such as zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and the clustered regularly interspaced palindromic repeat/CRISPR-associated protein 9 system (CRISPR/Cas9) have been popular for tailored modifications. Out of these, CRISPR/Cas9 tool holds great promise for crop improvement as it requires only the genomic sequence information, and works by introducing site-specific double-stranded breaks at the target location by the enzyme Cas9. These breaks are repaired by non-homologous end-joining (NHEJ) and homologous recombination (HR) repair pathways.

The CRISPR/Cas9-mediated genome editing has been successful in a number of horticulture crops including tomato (Brooks *et al*., 2014; Čermák *et al*., 2015; Pan *et al*., 2016; Soyk *et al*., 2017), potato (Wang *et al*., 2015), sweet orange (Jia and Wang 2014), apple (Nishitani *et al*., 2016), and cabbage (Lawrenson *et al*., 2015) for editing different genes responsible for different traits. This technique has opened up possibilities for designing various features not only in plants but also in insects. The pest resistance crops can be developed by changing the effector-target relationship and knocking out host susceptibility genes (Akbar *et al.*, 2023). In the present study, *phytoene desaturase* (*PDS*) gene (Solyc03g123760.2.1) has been edited using the CRISPR/Cas9 in tomato variety Ailsa Craig. The *PDS* encodes phytoene desaturase which is a key enzyme for carotenoid biosynthesis and its knock out or silencing would lead to photobleaching or albino phenotypes (Liu *et al*., 2002; Pan *et al*., 2016). The mutations were analysed in T_0 generation resulting in insertion or deletion mutations and the complete albino plants were

obtained. This successful *PDS* gene editing shows demonstration of genome editing in tomato which will serve as a basis for editing of other important genes such as *Pectate lyase* (*PL*) for enhancing the shelf life of important tomato commercial cultivars.

MATERIAL AND METHODS

Plant materials. Seeds of the Ailsa Craig tomato variety were obtained from Tokushima University, Japan. Seeds and fruits of tomato varieties: Florida 556, Roma, Punjab Gaurav, Punjab Ratta, Punjab Sartaj, Punjab Swarna, and PAU 2381 (pre-breeding line) were obtained from Vegetables Research Farm, Department of Vegetable Sciences, PAU. Plants of Tomato variety Punjab Ratta were grown in a greenhouse at 25-29 °C at 16h/8h (Light/Dark) cycle. Vegetative and fruit tissues were collected from greenhouse grown tomato plants and used for further analysis in lab facilities of School of Agricultural Biotechnology, PAU, Ludhiana.

sgRNA designing and confirmation. The *PDS* gene sequence (Solyc03g123760.2.1) from Ailsa Craig (https://solgenomics.net/) variety of tomato was used for sgRNA designing using web-tool focas (http://focas.ayanel.com) and CRISPOR tool (Concordet and Haeussler 2018).

Vector used. CRISPR/Cas9 vector, pEgPubi4_237-2A-GFP was prepared and obtained from Tokushima University, Japan. The single guide RNA (sgRNA) was cloned in this vector at Tokushima University, Japan by Dr. Prashant Mohanpuria. The empty vector was used as mock and sgRNA containing construct was used for transformation of tomato by leaf disc method in var. Ailsa Craig.

***Agrobacterium*-mediated genetic transformation of tomato leaf discs.** The seeds of Ailsa Craig were sterilized with 70 % ethanol for 2 minutes, followed by dipping in 4 % Sodium hypochlorite (v/v) solution containing 0.05 % Triton x-100 (v/v) and shaking for 45 minutes. The seeds were rinsed with distilled water and dried on sterilized filter paper in a laminar air flow hood. Tomato plants were allowed to grow from seeds under 16 h light/ 8 h dark cycle at 23^{o} C on tomato germination media (Basal MS media) for 7-10 days. The leaf discs (5-7 mm) were cut from the seedlings grown, dipped in *Agro* infection media for 30 min, and cultured on cocultivation media (MS media supplemented with 100 mM acetosyringone) upside down under dark conditions for 48 h. The leaf discs were then transferred to callus induction media (MS media containing 1 mg/L zeatin), and 3-5 mm callus was regenerated after 2-4 weeks of inoculation. The callus was screened using GFP marker to select the transgenic calli at the early stages. The transgenic calli were transferred to shoot induction media (MS media containing 1 mg/L zeatin) supplemented with 100 mg/L kanamycin (as plant selection) (Fig. 2) and finally to root-induction media (Basal MS media) followed by acclimatization on soil.

Detection of mutation and sequencing. The genomic DNA was isolated from the leaves and the T-DNA integration was confirmed using Pubi4 seq F3 (5'CTGGACTTTTTGGAGTTGACTTGA3') and At Cas9 115-96Rv (5'CGGTGTTTCCGAGAACCTTG3') primers. The PDS gene was amplified from the plants with SlPDSF1769-1796 (5'GGATTATCTTGCTTATGGAGTCACCAAG3') and SlPDS R2 199-2173 (5'GGTTCTGAATATTTGGGTAAGCCCCAA3') primers. The mutation was detected in the amplified PDS PCR product using Surveyor® mutation Detection Kit (Transgenomic, USA) following the manufacturer protocol. The samples were custom sequenced by Sanger sequencing (BioServe Biotechnologies, Hyderabad, India) for detection and analysis of mutations.

Shelf life of different tomato varieties. To assess shelf life of different tomato varieties, three fruits of red ripe developmental stage from each of the seven tomato varieties: Florida 556, Roma, Punjab Gaurav, Punjab Ratta, Punjab Sartaj, Punjab Swarna, and PAU 2381 were harvested from plant at Vegetables Research Farm, PAU and surface cleaned by washing in autoclaved distilled water. After drying of tomato fruit surface, each fruit was kept in a basket with good aeration and stored at room temperature (25°C and 55-60% relative humidity). Fresh weight of each fruit was taken on first day and the change in weight was measured for eight consecutive days until the fruits show signs of deterioration. From this, per cent loss in fresh weight of fruit was calculated and the variety having maximum per cent loss in fresh weight was selected for the shelf life improvement.

***Pectate lyase* (*PL*) genes of tomato, gene structure and chromosomal location.** The *PL* gene sequence of *Solanum lycopersicum* (tomato) was downloaded from the Sol Genomics network (http://solgenomics.net/). The fruit softening specific sequence was searched out of these *PL* gene sequences. The genome annotation files (genome feature files i.e., gff files) of *S. lycopersicum* was downloaded from the SGN database and schematic diagrams of the structure of *PL* genes in these species were prepared using TBtool (Chen *et al*., 2020). To determine the chromosomal location of *PL* gene family members, nucleotide sequences of the respective hits were extracted and used as input for mapping *PL* genes on chromosomes of the tomato (*S. lycopersicum*) using TB tool (Chen *et al*., 2020).

RESULTS AND DISCUSSION

A. Transformation of tomato with CRISPR vector (PDS) and regeneration of albino tomato plants

The sgRNA was designed from the *PDS* gene using the web-tool focas and CRISPOR (Fig. 1). In general, the sgRNA consists of crispr RNA (crRNA) fused to the (trans-activating crRNA (tracrRNA) and a 20-nucleotide sequence which is complementary to the target region. The crRNA guides the system to the matching sequences of DNA and the tracrRNA forms a complex with crRNA interacting with Cas9 for proper functioning of the CRISPR/Cas9 system and also plays a major role in the maturation of gRNA by mediating RNase III-dependent RNA processing (Liu *et al*., 2022). For the designing and selection of sgRNA, it is mandatory to take care that the exon's target region

should lie close to the terminal ends and preferably from the starting regions. This would result in the generation of cells with different start codons resulting in a frame-shift mutation. The designed sgRNA from the *PDS* gene was assembled into pEgPubi4_237-2A-GFP vector through golden gate cloning (Fig. 1).

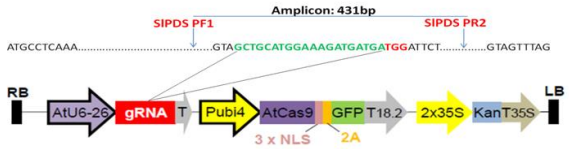

Fig. 1. Graphical representation of CRISPR/Cas9 construct pEgPubi4_237-2A-GFP for PDS gene of tomato and sgRNA designed from PDS gene.

Agrobacterium-mediated transformation of tomato leaf discs with pEgPubi4_237-2A-GFP construct resulted in generation of 1510 T_o tomato transgenic lines. For this about 550 leaf discs were transformed. From each of the tomato leaf disc, 3-8 shoots per callus were generated which showed positive GFP expression at preliminary screening (Fig. 2). Upon analysis of PDS gene editing, 20 % of the tomato transgenics (T_o population) showed complete albino phenotypes signifying the complete or partial loss of *PDS* gene function, around 40 % of the tomato transgenics were chimeric where leaves showed albinism to a variable extent with modified *PDS* gene function and 40 % of the tomato transgenics were green transformants without visible albino phenotype.

We selected the *Agrobacterium*-mediated transformation method which is inexpensive as compared to the other methods of transformation and is very popular in tomato. We generated 510 calli with a comparable transformation efficiency of 80 %. The calli were selected on kanamycin (100 mg/L) containing selection media which led to delayed shoot growth in the case of albino plants. This negative selection impact has also been reported in the case of crops like sugarcane (Rastogi *et al*., 2018) and cotton (Bibi *et al*., 2013).

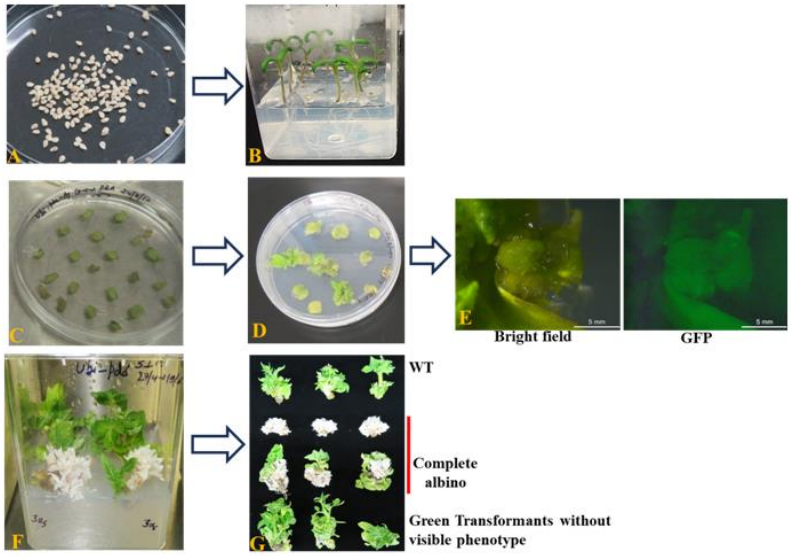

Fig. 2. Agrobacterium-mediated genetic transformation of tomato with CRISPR/Cas9 construct of PDS gene. A) Seeds of tomato variety Ailsa Craig. B) The 7-8 days old seedlings grown on basal MS media. C) Leaf disc cut from the seedlings and co-cultivated on MS media. D) Callusing and shooting induced from transformed leaf disc. E) Visual selection of transgenic callus under bright field and GFP fluorescence. F) Albino shoots generated on shoot induction media. G) Different categories of transformed tomato shoots produced from pds gene editing.

B. Detection and analysis of mutation in T_o generation transgenic tomato plants

The T-DNA integration was confirmed in 1210 transgenic tomato plants using Pubi4 seq F3 and At Cas9 115-96Rv primers (Fig. 3a). In these PCR-confirmed tomato transgenic plants, the mutations were first detected using Cel1-assay kit (Surveyor mutation detection kit) at the *PDS* target site (Fig. 3b) and further analyzed through Sanger sequencing of that site. The sequencing shows deletion of 2 bp at 3-4 bp upstream of PAM region of the complete albino plants (Fig. 3c). The InDels were not observed in all the PCR confirmed plants because there double stranded breaks were failed to take place and possible cellular repair mechanisms would work to repair the target regions of the gene. Wild type sequence is shown at the top with 20-nt guide sequence in red and PAM sequence in green. Deleted nucleotides are labelled as red and the number of mutated nucleotides is shown on right side of each sequence.

C. Analysis of shelf life of different tomato varieties

The fruits of tomato varieties: Florida 556, Roma, Punjab Gaurav, Punjab Ratta, Punjab Sartaj, Punjab Swarna, and PAU 2381 were collected at the red ripe stage from Vegetable Research farm, PAU, Ludhiana. These fruits were stored at room temperature (25 °C) and the loss of texture, integrity and decrease in per cent fresh weight was measured as compared to control. The fruits from all the varieties showed deterioration in shelf life i.e., loss of texture, and integrity of pericarp layer within eight days of storage.

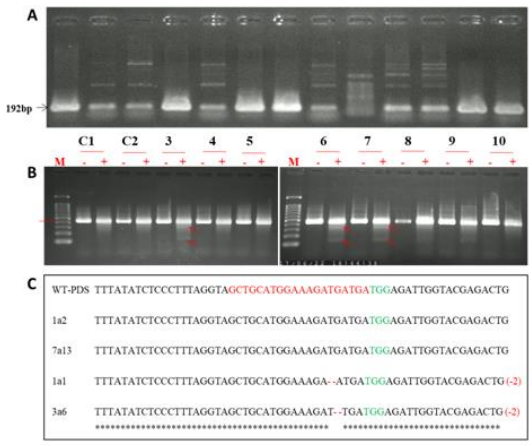

Fig. 3. Screening of PDS gene edited T_o generation tomato mutants. A) T-DNA (Cas9) integration confirmation using Pubi4 seq F3 and At Cas9 115-96Rv primers. B) Mutation analysis using Cel-1 assay on 2.5 % agarose gel; -: Cel-1 untreated sample, + : Cel-1 treated sample, C1: ubi control, C2: Wild-type PDS gene control. C) Representative mutations at target sites. On the left side, T_0 tomato plants are indicated.

The weight of the fruit was measured for eight consecutive days and per cent loss in weight was calculated for fruits of each variety. The per cent weight loss is an important parameter for shelf life assessment as it is attributed to mechanisms such as loss of respiration and loss of water (Sinha *et al*., 2019). The images of the fruits at 0 day and 8 days are represented in Fig. 4 B, as at 8 days fruits showed a wrinkled appearance in each of the varieties due to loss in texture and integrity. The maximum physiological loss of weight was appeared in variety Florida 556, the variety from the USA, while minimum physiological loss of weight was appeared in PAU 2381 (Fig. 4A). After Florida 556 the per cent loss in weight was greater in variety Punjab Ratta among all other varieties (Fig. 4A). The tomato variety Punjab Ratta is a determinate type resistant to late blight disease, has good TSS (4.9), and has good processing value but has low shelf life and thus, will be selected for further study.

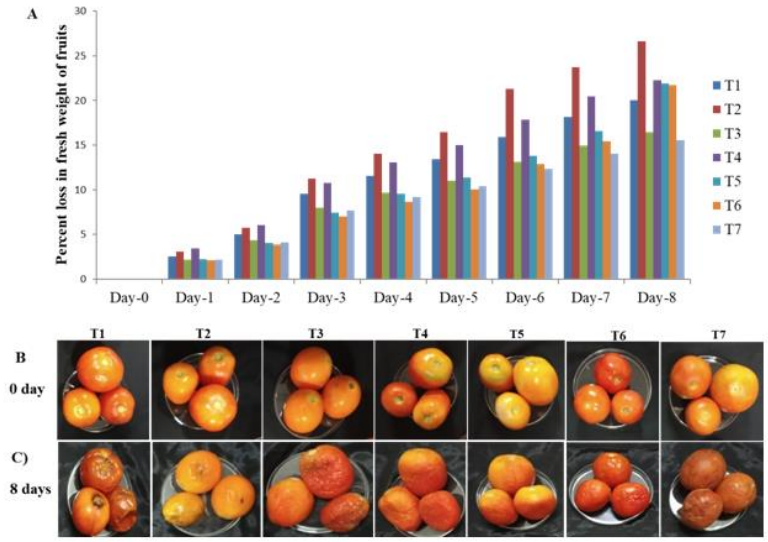

Fig. 4. Shelf life of different tomato varieties kept at room temperature (25 °C). (A) Physiological weight loss in seven tomato varieties, T1: Florida 556, T2: Roma T3: Punjab Gaurav, T4: Punjab Ratta, T5: Punjab Sartaj, T6: Punjab Swarna, and T7: PAU 2381 (pre-release line) from 0 to 8 days. Ripe fruits of these tomato varieties kept at room temperature at 0 day (B) and after 8 days (C) of storage.

In silico studies of *PL* genes

Gene structure, chromosomal location analyses of the *S. lycopersicum*. Although, five *PL* genes have been identified in *S. lycopersicum* through quantitative reverse transcription PCR (qRT-PCR) but during ripening only one allele (*Solyc03g11690*) was shown to have higher expression (Uluisik *et al*., 2016). Also, one report showed dominant expression of gene *Solyc03g111690* (fruit softening-specific) during fruit ripening of tomato variety 'Ailsa Craig' (Yang *et al*., 2017). Thus, *PL* (*Solyc03g111690*) was chosen as the candidate gene for the improvement of tomato fruit firmness or shelf life (Uluisik *et al*., 2016; Yang *et al*., 2017) in tomato variety 'Punjab Ratta'. The analysis of *PL* genes of *S. lycopersicum* revealed that introns were present in all detected *PL* genes. Gene structure analysis revealed high diversity in the exon numbers of *PL* genes in *S. lycopersicum* which could be related to the diversification of their functions. The exon numbers vary from 3-8 in the case of *S. lycopersicum*, while the candidate gene *Solyc03g111690* was found to be containing 4 exons and 3 introns (Fig. 5B). The second exon was the largest in case of candidate *PL* gene and thus, the sgRNA was designed from the exon-2 of *PL* gene i.e., the exon-2 region was selected as a target region for genome editing studies and this candidate gene target region will be further used for designing sgRNA which will target the *PL* gene of commercially important tomato variety Punjab Ratta for enhancing its shelf life. The chromosomal distribution of *PL* genes of *S. lycopersicum* was determined according to their genomic locations and gene features positions.

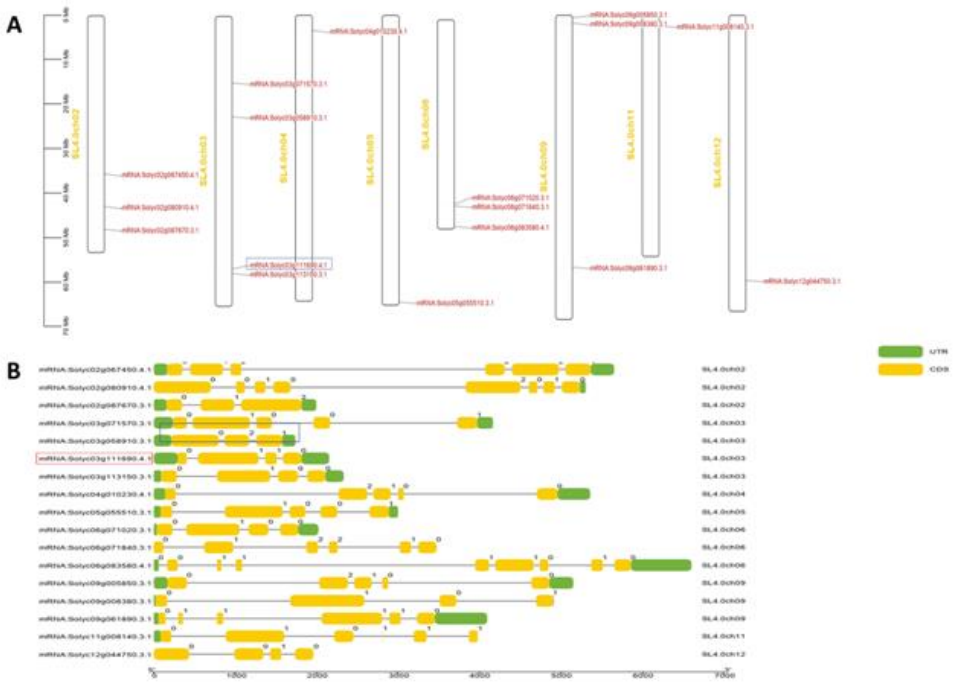

Fig. 5. *PL* gene structure analysis and chromosomal location prediction in *Solanum lycopersicum* A) Chromosomal location of *PL* genes in *S. lycopersicum* predicted using TB tool. In box: fruit softening specific candidate *PL* gene highlighted present on chromosome 3. B) *PL* gene structure analysis of *S. lycopersicum* using TB tool, the black lines, green boxes, and yellow-colored boxes represent introns, UTR regions, and exons, respectively. In red box: fruit softening specific candidate *PL* gene containing 4 exons and 3 introns.

In case of *S. lycopersicum* maximum no of *PL* genes were distributed on chromosome 3 anchoring 4 *PL* genes, followed by chromosomes 2, 6, and 9 containing 3 *PL* genes each (Fig. 5A). Rest of the genes were located on chromosomes 4, 5, 7, 11 and 12 while *PL* genes were absent on chromosome 1, 8 and 10. The candidate gene *Solyc03g111690* was also anchored on chromosome 3.

CONCLUSIONS

CRISPR/Cas9 technique has become voguish because of its adaptability, low cost, and versatility. Using modest constructs, we demonstrated the efficiency of this technique in editing *PDS* gene of tomatoes. To test the efficacy of this technique, the CRISPR edited albino tomato plants with complete or partial loss of *PDS* gene function were produced. This successful demonstration of the *PDS* gene of tomatoes has served as a basis for editing another target gene such as *PL* (for enhancing shelf life) in other tomato commercial varieties. We have explored the *PL* target gene from different tomato varieties and CRISPR/Cas9 constructs have been prepared using the PL gene.

FUTURE SCOPE

The CRISPR/Cas9 technique has successfully been established through the *PDS* gene, by using this technique the *PL* gene of the Punjab Ratta variety of tomato would be edited using the same procedure. For this, the sgRNAs have been designed from exon 1 and exon 2 and used for targeting the *PL* gene of the Punjab Ratta for shelf life improvement and the work is afoot. Thus, in coming future commercial tomato variety with the PL gene edits could be realized to enhance the shelf life of tomatoes in field conditions.

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Conflict of Interest. None.

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