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Codon optimization of chicken β *Gallinacin-3* gene results in constitutive expression and enhanced antimicrobial activity in transgenic *Medicago sativa L.*

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ABSTRACT

Gallinacin-3 (Gal-3) is a newly discovered epithelial beta-defensin that acts as cationic antimicrobial peptides, and plays an important role in chicken innate immunity. However, the gallinacin-3 precursor contained a lengthy C-terminal region, which often hindered its expression. After codon optimization of *Gal-3* and construction of an expression vector, the transgenic plants of *Medicago sativa* were obtained. Transgenic plants were validated and expression of proteins was detected. The antimicrobial activity of chicken β Gal-3 was analyzed and effects of chicken β Gal-3 on the body weight and intestinal microflora of mice were described. Our results demonstrated that the codon optimized chicken Gal-3 was stably expressed in transgenic *Medicago sativa* using the pCAMBIA3301 expression vector under the control of protein phosphatase (Ppha) promoter. Five transgenic plants with the highest expression of chicken β Gal-3 were selected, and were evaluated for the in vitro antimicrobial activity against *Escherichia coli, Staphylococcus aureus* and *Salmonella typhi.* Our findings confirmed that the Minimum Inhibitory Concentration (MIC) of the three bacterial strains were 32, 16 and 128 μg/mL, respectively. In addition, the effect of chicken Gal-3 on the body weight of mice fed with transgenic plants showed no significant deviation compared with that of the control group. Similarly, no loss of intestinal microflora was evident in the experimental group compared with the control group. Together, our findings demonstrate an alternative method for the stable expression of chicken Gal-3 with significant antibacterial effects and potential probiotics uses. In addition, this study may also be useful in the development of resistant *M. sativa* plants against pathogenic bacteria in future studies.

1. Introduction

Medicago sativa L. is a perennial legume forage known for its high nutritional value and exceptional palatability (Cen et al., 2020). It is widely grown as cattle feed all through the world and is rapidly gaining popularity in China in terms of farmed area (Li et al., 2011; Shi et al., 2017). Because of its important nutritional function in the maintenance of animal husbandry, it has earned the appellation of "the king of forage" among farmers. M. sativa L. is also renowned for its stress- and climate-resistance, since it can grow in a variety of climates and soil types, resulting in high adaptability, a long shelf life, and a high yielding capacity. Besides, it is a rich source of dietary fiber, edible protein,

Abbreviations: Gal-3, Gallinacin-3; Ppha, protein phosphatase; MIC, Minimum Inhibitory Concentration; ELISA, enzyme-linked immunoassay; Nm, nanometer.

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vitamins, beneficial minerals, saponins, flavonoids, carotenoids and other bioactive components (Cui et al., 2020; Li et al., 2020). Over the years, the widespread usage of *M. sativa L.* has gained significance in integrated agricultural systems, which include livestock industries, grassland ecological preservation, dairying, and the conversion of crops to forest and grassland (Shi et al., 2017). Thus, it is essential to produce new alfalfa varieties capable of meeting production requirements under local conditions, which highlights both possibilities and limitations for alfalfa breeding.

Defensins, a group of cationic, cysteine-rich antimicrobial peptides, are widely distributed among animals, plants, insects, etc., with a demonstrable role in their innate immune system. Based on their spatial structure, defensins can be categorized as plant defensins, insect defensins, α-defensins, β-defensins and θ-defensins. Their broad-spectrum antibacterial properties have been well described, which means they can act on both Gram-negative and -positive bacteria (including mycobacteria) (Yacoub et al., 2016). Their mechanism of antibacterial action is mainly attributed to their ability to permeate bacterial membranes by perforating the bacterial cytoplasmic membrane to form ion channels, causing leakage of intracellular water-soluble substances and ultimately death of bacteria (Jarczak et al., 2013; Dong et al., 2016). In animals and plants, defensins can be locally composed or differentially expressed, providing a stronger defense response to infection (Contreras et al., 2019). The first isolation of Chicken defensin from chicken neutrophils was reported by (Harwig et al., 1994), which include a total of 14 new chicken defensins, all of which are β -defensins. In China, chicken defensins have been mainly obtained from the tissues and organs of silky fowl and three-yellow chicken (Ji et al., 2008; Zhang et al., 2008).

β Gallinacin-3 (β Gal-3) is one of the important antimicrobial peptides exhibiting strong antibacterial activity against several pathogenic bacteria in plant. Considering its high drug resistance capacity of β Gal-3, we previously expressed the Chicken β Gal-3 gene in the Arabidopsis seeds demonstrated strong antibacterial activity against *Escherichia coli*, *Staphylococcus aureus* and *Salmonella typhimurium* (Jin et al., 2018). Another study investigated the efficient transformation of the chicken Gal-3 gene into M. sativa L. and proved the antibacterial activity of chicken Gal-3, but did not explore the feasibility of using such transgenic plants as a forage source (Jin et al., 2012). Because of the endogenesis capacity of β Gal-3, a few negative effects on the host system have been reported, therefore, transducing Gal-3 into the plant genome and cultivating transgenic plants as feed additives can be implemented to minimize chicken infectious disease. In this study, we tend to stably express the chicken β Gal-3 in *M. sativa L.* and determine its antimicrobial activity against Escherichia coli, Staphylococcus aureus and Salmonella typhi together with its impact on the body weight and intestinal microflora of mice ingesting water supplemented with chicken β Gal-3.

2. Materials & methods

2.1. Treatment of M. sativa. seeds

Plump seeds of *M. sativa L.* (Longmu 803 mutant No. 26) were obtained from the Ministry of Education Engineering Research Center of Bioreactor and Pharmaceutical Development, Jilin Agricultural University. The seeds were rinsed with distilled water for 1 h and then treated with NaCl solution (1.5% effective chlorine) for 15 min (with periodic overturning). After washing them five to six times, the seeds were soaked in sterile water for 24 h. Then, the seeds were taken out and placed in a culture dish with sterile wet filter paper. After germination, the seeds were inoculated on Murashige and Skoog medium and cultivated at 25 °C under 14 h light/10 h dark condition. On reaching the desired maturity, compound leaves were used as explants for agrobacterium infiltration and transformation.

2.2. Codon optimization of chicken β Gal-3 encoding gene

Based on the codon usage characteristics of *M. sativa* (https://www.kazusa.or.jp/ codon/ index.html), the chicken β Gal-3-encoding gene available under the GenBank accession no. (NP_989981) at NCBI was optimized using DNAWORKS3.0 (http://mcl1.ncifcrf.gov/dnaworks/) and Synthetic Gene Designer software without changing its amino acid sequence. The optimized nucleotide sequence β Gal-3 was then screened using the Genscript Rare Codon Analysis Tool (http://www.genscript.com.cn/rare_codon_ analysis_ tool.html, Shanghai Jierui Biotechnology Co., Shanghai, China), in order to record its GC content and distribution, CAI value, optimal codon usage frequency and repeat sequence domain. An optimized codon was also synthesized by Shanghai Jierui Biotechnology Co., which was cloned into pEASY-Gal3 plasmid.

2.3. Construction of expression vector pCAMBIA3301-Gal3

The linearized plant expression vector pCAMBIA3301 was subject to double restriction digestion using a pair of restriction endonucleases *Nco*I and *BstE*II (Fig. 10). After digestion, the large fragment was recovered and purified using the EasyPure Quick Gel Purification Kit (TransGen Biotech Co., Beijing, China). The primer pair of "*Gal-3* recombinant F" and "*Gal-3* recombinant R" were designed according to the synthesized *Gal-3* gene sequence using Primer5.0 software, and 15-bp homologous sequences were added to the 5' ends of the two primers. The amplification of *Gal-3* recombinant fragment was carried out using the following PCR condition: (2 PowerTaq PCR master-mix 10 μL, template DNA 1 μL, upstream and downstream primers 1 μL (10 μmol/μL), adding ddH2O to 20 μL, the amplification reaction conditions were as follows: 98 pre-denaturation 5 min; 94 denaturation 60 s,52 annealing 45 s, with 72 extension for 1 min and 24 cycles; PCR products were taken for 10 g/L agarose gel electrophoresis after 72 min extension). The homologous fragment of *Gal-3* recombinant F came from the end of pCAMBIA3301 cut by *Nco*I, and that of *Gal-3* recombinant R was from the end of pCAMBIA3301 cleaved by *BstE*II. The expression vector pCAMBIA3301-Gal3 was prepared using the CloneEZ® recombinant cloning kit (GenScript, Wuhan, China). The recombinant vector was then transformed into the competent cells (*EHA105*) of *A. tumefaciens* using freeze thaw method.

2.4. Genetic transformation of M. sativa L.

The genetic transformation of *M. sativa* with β Gal-3 gene was carried out under control conditions. At first, the compound leaves of *M. sativa* were sliced into two parts and then subject to inoculation on a pre-culture medium. After allowing the culturing under continuous light for 7 days, the explants were then immersed in the *A. tumefaciens* dip solution containing the expression vector pCAMBIA3301-Gal3 for 10 min. After discarding the bacterial solution, the explants were placed on a co-culture medium supplemented with TM-1 + 2,4-D (2.0 mg/L) + 6-BA (1.0 mg/L) + NAA (0.1) mg/L for 3 days in the dark environment. Then, after washing thrice with an MSO medium containing 250 mg/L cefuroxime and 250 mg/L carbenicillin, the explants were dried with sterile filter paper and inoculated on an antibacterial medium. The explants were cultured at a condition of 16 h light/8h dark condition for 7 days and sub-cultured once every 2 weeks. On the 20th day when embryoid germination was obvious, the regenerated shoots were cut off and inoculated in a rooting medium. Finally, the leaves of T1 generation having a radius of about 0.5 cm were collected and then grounded thoroughly. A total volume of 200 μL protein dissolved liquid was mixed and a bar strip was placed downward and read after 5 min. The bar strip reading of the transgenic plant was compared with that of wild-type plant of *M. sativa.*

2.5. Screening and detection of transgenic plants

For screening and detection of transgenic plants, the total DNA content was extracted from the leaves of transgenic and wild-type plant. The initial confirmation was performed with the PCR amplification of marker *bar* gene and target *Gal3* gene using "bar detection F" and "bar detection R." primers and "P35-Gal3 detection F" and "P35-Gal3 detection R," primers respectively. The primer pairs were designed using the Premier 5.0 software. In addition, southern blot hybridization was performed to confirm the presence of single copy Gal3 in transgenic plants. The DNA from wild-type and transgenic plants was collected and subject to probe F and probe R as primers in the PCR system. The EasyPure PCR purification kit (TransGen Biotech, Beijing, China) was used for product recovery following the manufacturer's instructions. The recovered product was then subject to the process of labeling of the target gene fragment. A 200 μL trans 8 k marker was also recovered and stored in the refrigerator at −20 °C for later use. Moreover, a one-step RT-PCR was performed to confirm the transcription of Gal3 in the transgenic plants using the template RNA obtained from the wild-type and transgenic leaves with the help of TransScript One-step RT-PCR SuperMix kit (TransGen Biotech, Beijing, China). For the detection of transgenic *M. sativa*, "RT-Gal3 detection F" and "RT-Gal3 detection R" were used as primers.

2.6. Identification of plants with high expression of chicken β Gal-3

The leaves from transgenic and non-transgenic plants were grinded with liquid nitrogen and placed into centrifugal tubes. The total soluble proteins (TSPs) were then extracted from the leaves of both types of plants. Then, 1 mL of PBS buffer solution (137 mmol/L NaCl, 2.7 mmol/L KCl, 10 mmol/L Na_2HPO_4, 2 mmol/L KH_2PO_4) was added to each centrifugal tube at a ratio of 1:2 (w/v). The centrifugal tubes were placed on ice until complete meltdown, and then oscillated for 30 s followed by centrifugation at 15000*g* for 20 min at 4 °C. The supernatant was collected as TSPs and then mixed with the PBS buffer solution until it reached a concentration of 5 mg/mL. A filter paper with a diameter of 5 mm and a thickness of 1 mm, which had been heat-sterilized, was soaked in the previously prepared solution. Finally, the ratio of the TSP from five individual transgenic plants were compared with the wild-type plant.

2.7. Isolation and purification of chicken β Gal-3 protein

The TSP content extracted previously from transgenic plants were filtered using a 0.22 μm membrane and then placed in the ultrafiltration centrifugal tube with a cut-off molecular weight of 10 kD and centrifuged at 4000*g* for 15, 30, 45, 60 and 90 min. Then the effluent was collected and placed in another ultrafiltration tube with a cut-off molecular weight of 3 kD and centrifuged at 5000*g* for 15, 30, 45, 60 and 90 min. The concentration of each soluble protein was determined by Branford method. For the detection of chicken β Gal-3 in TSPs, the ELISA method was used which includes (commercial mouse anti-chicken β -defensin Gal-3 was used as the primary antibody, and horseradish peroxidase labeled rabbit anti-mouse was used as the secondary antibody. The color was displayed in freshly prepared TMB solution. Finally, the absorbance value was read at 450 nm and the content of chicken β -Gal3 antimicrobial peptide in the solution was calculated according to the standard curve). Based on the purity and recovery rates of chicken β Gal-3 from different ultrafiltration centrifugation solutions, the optimal recovery strategy was carried out. The detection of the target β Gal-3 protein was conducted using the combination of 15% Tricine SDS-PAGE and further purification was performed with western blotting.

2.8. Western blot hybridization

Rinse the membrane once with TBST (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05 percent Tween 20) for 5 min at room temperature. Prevent non-specific membrane binding for 1 h on a vibrating surface at room temperature. Followed by incubation, the membrane with a chosen primary antibody dissolved in TBST and 5% nonfat dry milk overnight at 4C. TBST three times for 5 min each. Incubate for 1 h at room temperature with OriGene's HRP-conjugated secondary antibody. Repeat three times washing for a total of five minutes each time with TBST. To the membrane, add the combined detection solution followed by 1 min incubation. Eliminate any remaining solution and wrap the membrane with clear plastic. Place the folded blot in an X-ray film cassette with the protein side facing up. Top the membrane with a layer of X-ray autoradiography film. For 15 s to 1 min, close the cassette. Remove the film to allow it to develop. More films may be added if necessary for short or long durations.

2.9. Antimicrobial activity of chicken β Gal-3

In order to investigate the antimicrobial activity of β Gal-3, a total concentration of 1 mL *E. coli*, *S. aureus* and *S. typhi* was spread on individual LB solid medium containing tryptone, yeast extract, NaCl. Filter papers with a diameter of 5 mm and a thickness of 1 mm, which had been pre-soaked in pure chicken Gal-3, were pasted onto each plate for overnight incubation at 37 °C. The next day, the diameter of inhibition zone was measured to determine the content of antimicrobial peptides presented in the leaves of transgenic plants, which is a direct indicator for an estimation of chicken β Gal-3 expression. After diluting the purified chicken β Gal-3 with 10 mM sodium phosphate solution (pH 7.4) by double dilution method, a 100 μL of the solution was placed into a 96-well plate and an equal volume of bacterial solution (10^6 cells per milliliter) was added to each well. After incubation at 37 °C for 12 h, the value of OD_{600} absorbance was determined using an enzyme-labeled instrument (SPARK, TECAN, Switzerland) for calculating the antimicrobial activity.

2.10. The investigation of Gal-3 on the body weight and intestinal microbiota of mice

To examine the effect of Gal-3 on the body weight of mice, a total of Twenty KM mice having body weight (18–20 g) were divided into experimental group 1 and control group 1, ten in each group. Mice in experimental group 1 were fed with transgenic *M. sativa* plants, while those in control group 1 were fed wild-type plants. For up to 30 days of continual feeding, the body weight of each experimental group was measured on daily basis. Similarly, for the investigation of intestinal microbiota of mice, 20 KM mice (body weight 18–20 g) were divided into experimental group 2 and control group 2, ten in each group. Sterile drinking water containing 16 μM chicken β Gal-3 was provided to experimental group 2, and that containing 16 μM kanamycin was given to control group 2. A week later, the composition of mice intestinal flora was determined. All experimental conditions and design were authorized by the ethical committee of Laboratory Animal Quality Testing Center in Jilin Province (20190520004).

2.11. Statistical analysis

Data are presented as mean ± SE. Statistical comparisons were carried out using Tukey–Kramer and *t*-tests.

3. Results

3.1. Codon optimization of Gal3 gene encoding chicken β Gal-3 peptide

In order to perform codon optimization of *Gal3* gene, the code adaptation index of *Gal3* gene from chicken was increased from 0.59 to 0.94 (Fig. 1), while its GC content was decreased from 49.72% to 42.39% according to the codon bias of *M. sativa* (38.94%). The GC

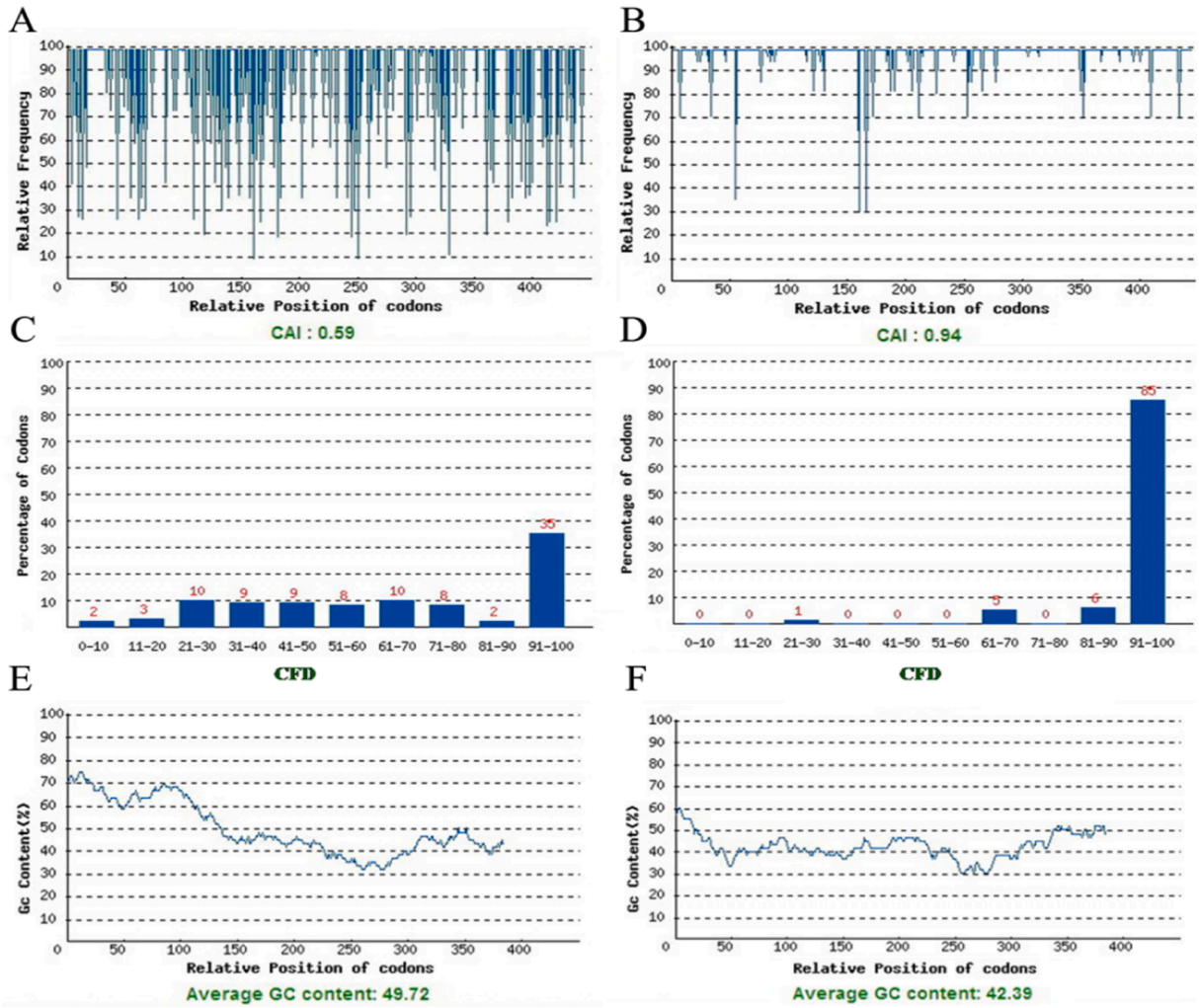

Fig. 1. Codon optimization of chicken *Gal3* gene according to according to the codon bias of *M. sativa*.

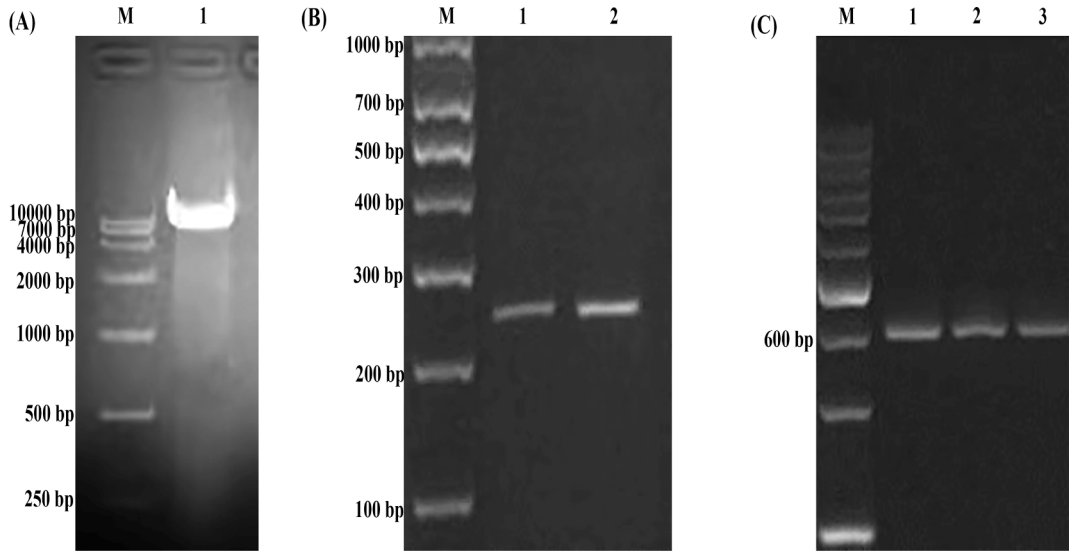

Fig. 2. Construction and validation of recombinant pCAMBIA3301-*Gal3* vector. (A) Detection of recombinant pCAMBIA3301 plasmid where M represents 10 K DNA marker and lanes 1 showed the size of pCAMBIA3301 plasmid (B) Amplification of the *Gal3* gene (250 bp) where M represents 1000 DNA ladder and lanes 1–2 represent the amplified products of *Gal3* (C) The combined amplification of the Ppha promoter and *Gal3* target gene in the recombinant plasmid where M represents 15 K DNA marker and 1–3 represent the target bands.

contents of the first, second and third bases were 46.54%, 38.78% and 31.51%, respectively. Moreover, the distribution was uniform, with no obvious GC or AT enrichment regions. The optimal codon frequency distribution was 91–100%.

3.2. Generation of the pCAMBIA3301-Gal3 expression vector

The expression vector of pCAMBIA3301 was primarily digested into two fragments using *NcoI and BstEII* restriction enzymes, of which the larger one was chosen to act as linearized carrier fragment 1 for ensuring a seamless connection (Fig. 2A). Similarly, a stable band of approximately (250 bp) consistent with the expected size of *Gal3* was amplified using the synthesized Gal3 specific primers. Furthermore, the insertion of the promoter *Ppha* and target gene *Gal3* into the expression pCAMBIA3301 vector was confirmed on 1 % agarose gel by comparing the combined size of the *Ppha* promoter and *Gal3* gene (Fig. 2C). The recombinant vector construct was further confirmed with sanger sequencing for base mutation.

3.3. Generation and screening of transgenic M. sativa plants

The pCAMBIA3301-Gal3 plasmid was introduced into *M. sativa* using the agrobacterium-mediated transformation system. A total of 3142 transgenic *M. sativa* lines were generated in this study. Beginning with pre-culturing up to the regeneration of transgenic *M. sativa* plants, an illustration of process flow is presented in (Fig. 3A). The results of PCR and bar strip test were used to screen the positive transformants indicating the stable integration of target gene into *M. sativa* plants (Fig. 3B, and 4A). Further confirmation was carried out with the amplification of the selectable marker gene (Bar) using the genomic DNA extracted from the transgenic plants (Fig. 4B). Furthermore, the expression analysis of chicken β Gal-3 at transcriptional level also confirmed the highest expression in A91, A375, C19, D57 and E245, respectively suggesting that the transcript of *β Gal-3* gene could be efficiently transcribed in transgenic lines (Fig. 5A). In addition, the southern blotting hybridization also confirmed the presence of *β Gal-3* single-copy in all five transgenic plants, except D57, indicating the stable transformation (Fig. 5B).

3.4. β Gal-3 protein expression in transgenic M. sativa

The quantification of β Gal-3 protein expression was carried out using the total soluble proteins (TSPs) obtained from the leaves of non-transgenic (control group) and transgenic plants (experimental group). Five selected transgenic plants were used as experimental group to measure the TSP content. It was found that the expression of chicken β Gal-3 was higher than that in wild-type plants. The highest expression of chicken β Gal-3 was detected in C19 transgenic line resulting in 4.76 mg/g*FW, accounting approximately 0.26% of the total TSP content. Similarly, the ratio of chicken β Gal-3 in other transgenic lines demonstrated 0.17% (A91), 0.07% (A375), 0.04% (D57) and 0.05% (E245), respectively. These findings suggested the increased protein expression of β Gal-3 in transgenic *M. sativa* compare to wild-type plants, and could be used as a rich source of active probiotics and other applications (see Fig. 6).

3.5. Development of the purification system of chicken β Gal-3 protein

The purification of β Gal-3 from the TSP content extracted from the leaf tissues of the five selected transgenic plants was carried out using a 10 kD and 3 kD ultrafiltration centrifugal tubes, respectively at different time points (15, 30, 45, 60 and 90 min.). The quantity of the purified chicken β Gal-3 protein was measured on the basis of retention portion of the effluent and the concentration of each soluble protein was determined by Branford method. After thorough filtration, the target protein of β Gal-3 with an expected size of 9 kD was initially separated on 15% Tricine SDS-PAGE. In addition, the soluble protein of β Gal-3 was further quantified by western blot hybridization indicating the high protein purity and recovery efficiency detection of the target protein component of chicken β Gal-3 in selected transgenic plants.

3.6. Inhibitory effects of chicken β Gal-3 on E. coli, S. aureus and S. typhi

The antimicrobial activity of β Gal-3 protein expressed in transgenic *M. sativa* against *E. coli, S. aureus and S. typhi* bacterial strains was investigated in comparison to the total protein content of wild-type *M. sativa*. The results of MIC values indicated that β Gal-3 significantly formed inhibitory zones against three bacterial strains but not in wild-

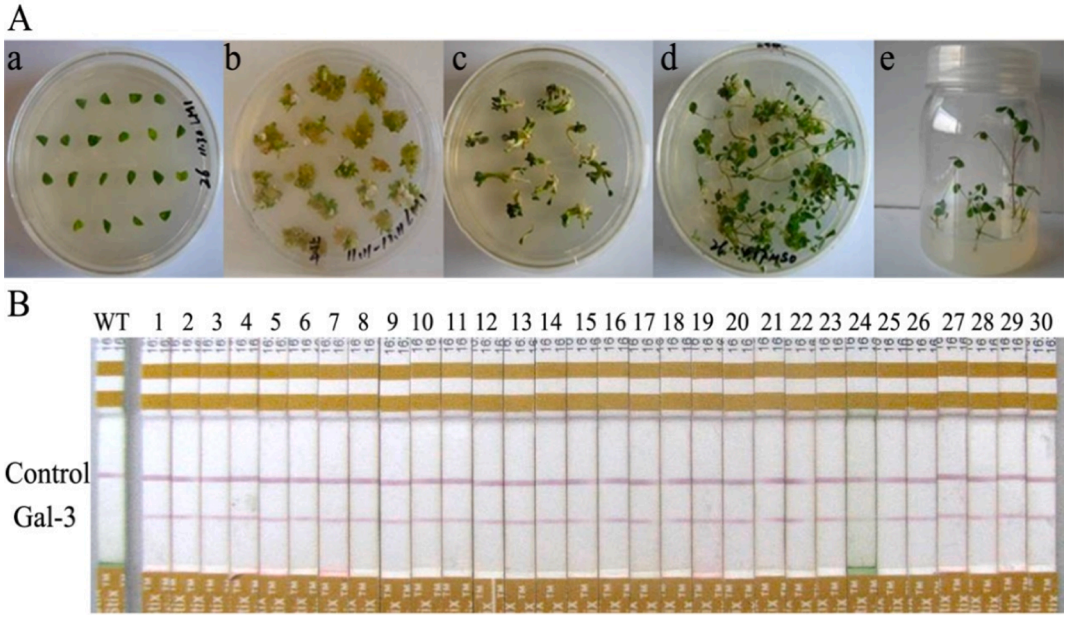

Fig. 3. Agrobacterium-mediated transformation of *β Gal-3* gene into *M. sativa.* (A) a, pre-culturing of the explant on MS medium; b, callus induction and infiltration with agrobacterium harboring *β Gal-3*; c, the development and screening of the embryoid; d, preparation of the differentiation culture; e, the establishment of rooting culture. (B) The illustration of the Bar strip test of the transgenic *M. sativa* resistant plants in comparison to the control plants.

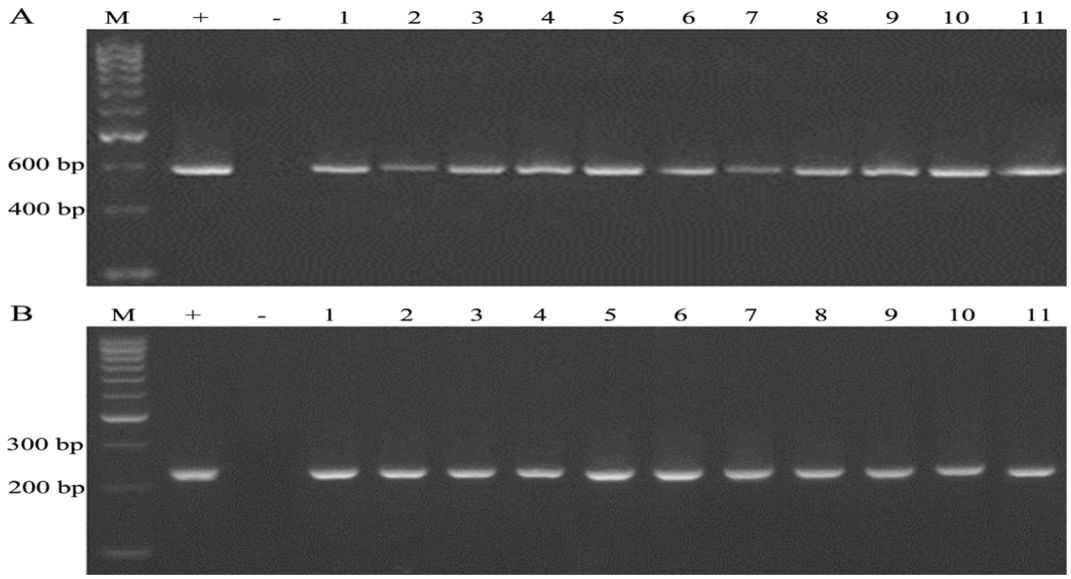

Fig. 4. PCR validation of Gal3 positive transformants using gene specific primers and selectable marker gene (Bar). (A) The amplification of the target fragment containing Gal3 gene and Ppha promoter. M: 1000 bp DNA marker; lanes 1–11: positive transformants. (B) The amplification of bar marker gene; M: 200 bp ladder DNA marker; lanes 1–11: positive transformants.

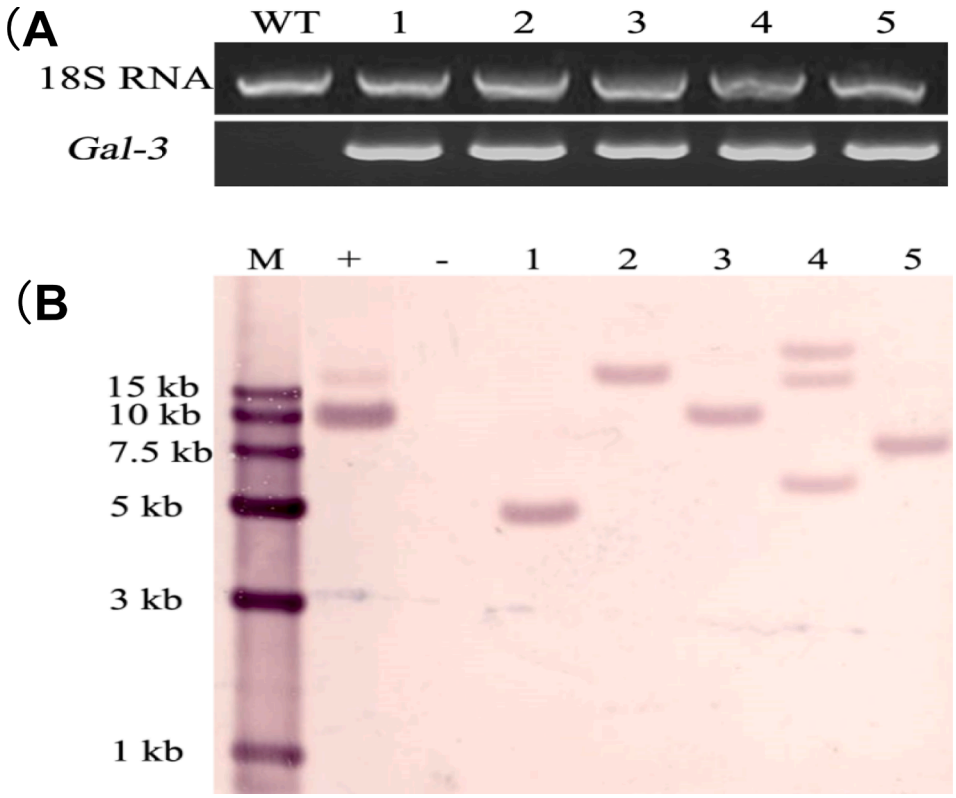

Fig. 5. The RT-PCR analysis and southern blot hybridization of transgenic plants harboring Gal3 gene. (A) the RT-PCR result of five selected transgenic plants where WT represents wild type; and lanes 1–5 represents transgenic lines of *M. sativa* including A91, A375, C19, D57 and E245, respectively. The 18 s Ribosomal RNA gene was used as internal control. (B) Southern blot analysis of the five selected transgenic plants. M, 15 k. lanes 1–5 denotes A91, A375, C19, D57 and E245, respectively.

type suggesting the greater potency of β Gal-3 as antimicrobial peptides. The MIC values of chicken β Gal-3 against *E. coli* (Fig. 7A), *S. aureus* (Fig. 7B), and *S. typhi* (Fig. 7C) were in the range of 32, 16 and 128 μg/mL, respectively. However, there was no obvious inhibitory zones produced in case of wild-type. These findings imply that Gals 3 may serve as an active antimicrobial agent and are a necessary component of the chicken host's innate immune system.

3.7. The effect of chicken β Gal-3 on body weight and intestinal microflora of mice

The body weight and intestinal microflora of mice fed with wild-type and transgenic *M. sativa* expressing β Gal-3 was further investigated for a period of continuous 30 days after the treatment. The effect of β Gal-3 on mice body weight resulted in a gradual increase ranging from 0.167 ±

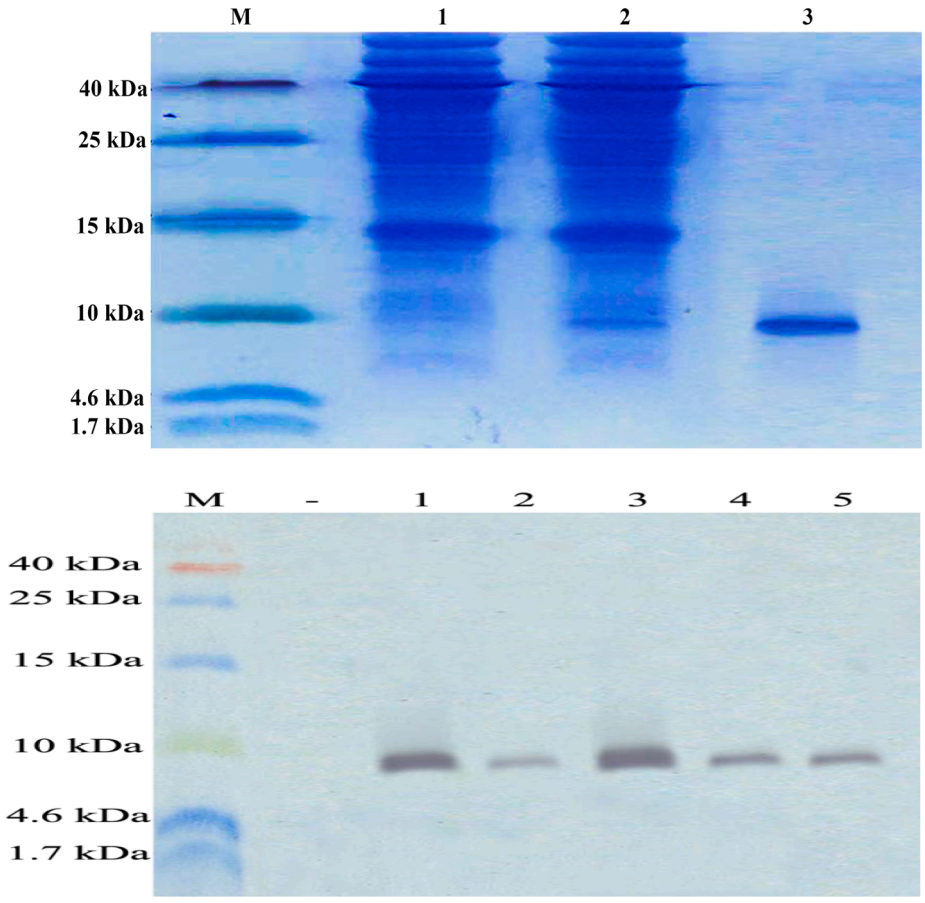

Fig. 6. SDS-PAGE analysis and western blot hybridization of β Gal-3 encoding protein in the selected transgenic plants. M: thermo low-range marker; lanes 1–5: A91, A375, C19, D57 and E245, respectively.

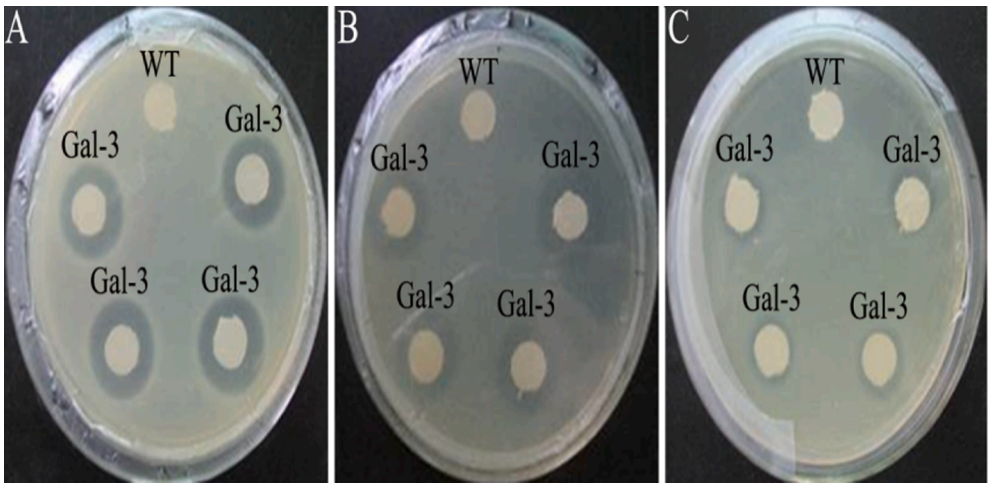

Fig. 7. Antimicrobial activity of chicken β Gal-3. (A). *Escherichia coli*. (B). *Staphylococcus aureus*. (C). *Salmonella typhi*. WT: total protein of wild type *M. sativa L.* Gal-3: purified chicken β Gal-3 at a concentration of 1 mg/mL.

0.085 g/day fed with transgenic *M. sativa* and 0.248 ± 0.038 for wild-type plants, respectively. These findings suggested a lack of statistically significant variability among the wild-type and transgenic plants (Fig. 8). In addition, the investigation of the intestinal microflora of mice supplemented with drinking water with chicken β Gal-3 showed no noticeable effects ($P > 0.05$) (Fig. 9). However, when supplementing water with kanamycin, a significant loss of intestinal microflora in mice was evidenced ($P < 0.05$). These findings provide significant evidence on the safety and use of β Gal-3 as forage ingestion in the poultry industry.

4. Discussion

Antimicrobial peptides have been shown to play a pivotal role in the innate immune system and to have broad-spectrum antibacterial (Grishin and Sokolov, 2014), antiviral (Tamamura et al., 1995), and

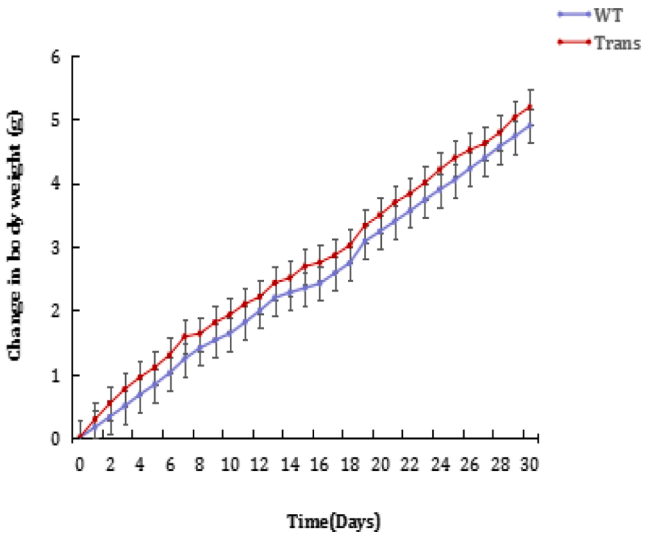

Fig. 8. The effect of β Gal-3 on the body weight of mice fed with transgenic *M. sativa* and wild-type plants.

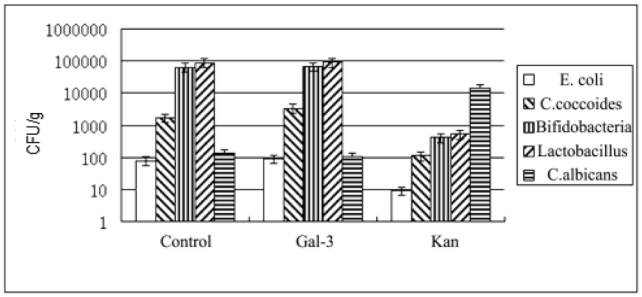

Fig. 9. Influence of chicken β Gal-3 on the intestinal microflora of mice.

anticancer (Baker et al., 1993) properties. However, due of the poor yield, low expression and presence of impurities, the wider use of antimicrobial peptides has been restricted on a large scale. Alternatively, the plant system can be utilized as bioreactor for the efficient expression of antimicrobial peptides. Several studies showed disease resistance potential by modulating the rapid expression of antimicrobial peptides in plants (Rivero et al., 2012; Shukurov et al., 2012) In this study, the codon optimized chicken β Gal-3 gene was constitutively produced in *M. sativa*, which showed significant antimicrobial activity against *E. coli*, *S. aureus* and *S. typhi.* In addition, the body weight of mice fed with transgenic *M. sativa* was comparable to that of the control group. In terms of the intestinal microflora of mice, supplementing drinking water with chicken β Gal-3 showed no visible interference, whereas the use of antibiotic alone demonstrated significant loss of intestinal microflora. To the best of our knowledge, this is the first study to address the safety of transgenic *M. sativa* forage ingestion on the intestinal microflora of mice.

Due to the difficulties of integrating animal-derived genes into practice, the deployment of these genes in plants might be quite proactive. The production of a target protein can be enhanced via codon modification, which results in rendering the gene compatible with plant characteristics without modifying the sequence (Liénard et al., 2007; Miriam and Laxa, 2017). Here, we developed the core sequence of chicken β Gal-3 by codon modification, with an optimal codon frequency distribution of 91–100%, indicating that it can be optimized for high expression in *M. sativa* leaves. According to previous studies, single-copy transformants can effectively avoid differences in expression caused by copy numbers during gene integration. Similarly, researches have shown that the complete single-copy plants have relatively small differences in gene expression levels (Shingo et al., 2005). We also screened five individual transgenic plants of *M. sativa* containing the optimized chicken β Gal-3 with the highest expression. The results of RT-PCR and southern blotting confirmed that the target gene could be normally transcribed in leaves, four of them being single copy, which could be stably inherited into the next generation. Although, our previous work demonstrated the generation of transgenic *M. sativa* plants with chicken β Gal-3, however, they were not single-copy plants and also did not show any feasibility to be used as forage (Jin et al., 2012). In the same way, the transformation of *Gal-3* in *A. thaliana* revealed the highest expression of β Gal-3 comprising 0.27% (4.76 mg/g) in the TSPs extracted from the seeds (Jin et al., 2018). In view of this, consistent with the our previous study (Jin et al., 2012), the finding of this research also demonstrated the highest expression of chicken β Gal-3 accounting 0.26% (4.76 mg/g) of the TSPs extracted from the leaves of transgenic *M. sativa* plants. Taken together, our data showed that chicken Gal-3 might be an excellent candidate for transgenic expression in plants.

Electrostatic interaction between antimicrobial peptides and structures on the bacterial surface is the most evident method for attracting the peptides to the bacterial membrane (Peschel, 2002). Gal-3 possesses wide ranging antiviral, antiseptic, and immune-stimulatory properties [26]. Recently, 14 distinct forms of Gal-3 have been identified in chickens and are designated as AvBD1–AvBD14 [27]. Gal-3 could be used as an immunological stimulant, feed additive, and antimicrobial agent in livestock production. Furthermore, traditional antibiotics may also be replaced by Gal-3 due to its non-toxicity, non-drug resistance, lack of environmental impact, and lack of drug residue. In our analysis, chicken β Gal-3 showed obvious antibacterial effects against *E. coli*, *S. aureus* and *S. typhi.* The MIC values of chicken β Gal-3 produced in transgenic *M. sativa* were in the range of 32, 16 and 128 μg/mL, respectively, which are consistent with the previous findings of (Jin et al., 2018) and (Fu et al., 2013). Importantly, the incidence of amino acid substitution, improper folding or incorrect disulfide bond formation during protein expression have been shown to interfere or even inhibit the biological activity of β gal-3 (Lay et al., 2003). However, the expression and activity of chicken β Gal-3 in our transgenic plants was unaffected by these factors and considered normal. On the other hand, the use of antibiotics against bacterial and fungal diseases in farmed animals is ubiquitous and their harmful residues is a matter of concern to human health (Pieri et al., 2020; Uddin et al., 2020). Therefore, the development of safer antimicrobial need more robust and stable alternative strategies. Our research adds to the existing evidence on the potential of chicken β Gal-3 as a genetic target for developing disease-resistant crops. More research in this direction could open many doors for developing safe ways of combating human and animal pathogens (Cools et al., 2017; Islam et al., 2017; Parisi et al., 2018). Although, we have tested the transgenic plant materials on mice models for toxicity, allergies, and possible horizontal gene transfer to the environment or to other species. However, we still believe that the long-term implications of using these GM plants as potential probiotics needs proper legislative framework to register them as new GM products in the future.

5. Conclusions

The stable expression of chicken β Gal-3 was developed in the transgenic *M. sativa.* plants following the codon optimization strategy. The significant antibacterial effects against *E. coli, S. aureus and S. typhi* were confirmed by measuring the MIC values in comparison to wildtype plants. Additionally, there was no change detected in the body weight of mice with feed containing transgenic *M. sativa* plants compare to wild plants. Moreover, of the intestinal microflora of mice, supplementing drinking water with chicken β Gal-3 showed no visible interference in comparison to kanamycin antibiotic. Altogether, the current work demonstrated the establishment of an alternate method for the sustainable production of chicken Gal-3, which has antibacterial and probiotic properties. In addition, this work may be valuable in the

production of M. sativa plants that are resistant to harmful bacteria in future research.

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CRediT authorship contribution statement

Libo Jin: Conceptualization, Methodology, Software, Supervision. **Yunpeng Wang:** Conceptualization, Methodology, Software. **Xiuming Liu:** Data curation, Writing – original draft. **Renyi Peng:** . **Sue Lin:** . **Da Sun:** Writing – review & editing. **Hao Ji:** Software, Validation. **Lei Wang:** Visualization, Investigation. **Yuting Zhang:** Visualization, Investigation. **Naveed Ahmad:** Data curation, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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