

Original Research Paper

Diosgenin Biosynthesis in the Sprouts of Fenugreek as Influenced by Chitosan

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Abstract: *Trigonella foenum-graecum* L. (Fabaceae), known as Fenugreek is an annual herb, contains various secondary metabolites including steroidal saponins, flavonoids, phenolic compounds and alkaloids. This study was planned to investigate the effect of concentrations of chitosan on growth, expression of diosgenin biosynthetic genes and diosgenin accumulation in sprouts of *T. foenum-graecum*. Results revealed that there is no positive response of chitosan in any of the studied growth parameters i.e. shoot and root length, fresh and dry weight. In control treatment without any chitosan solution the highest values for all the parameters were observed. The results indicated that increasing concentrations of chitosan had no significant effect on shoot and root lengths. The fresh weight of sprouts treated with chitosan decreased markedly, but not significantly, compared with that of the control. However, the dry weight of sprouts treated with chitosan was almost similar to that of the control. Diosgenin biosynthetic pathway in response to different concentration of chitosan enhanced to an increasing level in the expression of all treated sprouts than those of no chitosan (control) treated sprouts. The highest expression level was detected when treated with 0.05 mg/mL chitosan in most of the genes except *TfSQS*. The expression of *TfSQS* was slightly higher in sprouts treated with 0.01 mg/mL chitosan than in those treated with 0.05 mg/mL chitosan. The highest expression level was in *TfCAS* at 0.05 mg/mL chitosan exhibiting 3 times higher expressing than that of control. The increasing expression trend for both *TfSQLE* and *TfSTRL* remained up to 0.05 mg/mL chitosan showing 1.7 times more expression in this concentration and then started to decrease the expression level. Diosgenin content in the *T. foenum-graecum* sprouts did not respond positively to chitosan treatment. Our findings could be potentially implemented for future studies in this arena.

Keywords: Chitosan, Diosgenin, Fenugreek Sprouts, Gene Expression

Introduction

Genus *Trigonella* includes 75 species. The name comes from the Latin *Trigonus*, which means “three-angled”, in reference to the small, triangular appearance of the flower (Christen, 2002). This is known as Fenugreek, *Trigonella foenum-graecum* L. (Fabaceae), is an annual herb (Snehlata and Payal, 2012) cultivated widely from Iran to Northern India, as well as in Egypt, Southern Europe, China, West Africa, Pakistan and the Mediterranean countries.

One of the saponins present in fenugreek is diosgenin, a major bioactive chemical compound structurally similar to cholesterol and other steroids (Rahmati-Yamchi *et al.*, 2014). In the pharmaceutical industry, diosgenin is the principal precursor compound in the manufacture of several synthetic steroidal drugs (Chen *et al.*, 2015). It also represents a promising bioactive biomolecule that exhibits various biological properties; these include hypolipidemic, hypoglycemic, antioxidant, anti-inflammatory and antiproliferative activities (Jesus *et al.*, 2016).

Chitosan is a chitin derivative, a biopolymer characterized by unique properties, such as bioactivity, biocompatibility and non-allergenicity (Salachna and Zawadzńska, 2014; Malerba and Cerana, 2016). Chitosan acts in controlling plant pathogens, enhancing germination index, reducing germination time, increasing shoot height, root length, shoot and root dry weights (El Hadrami *et al.*, 2010) and eliciting natural defense response mechanisms (Walker *et al.*, 2004).

In this study, we investigated diosgenin biosynthesis-related gene expression and diosgenin content in fenugreek sprouts with chitosan treatment.

Materials and Methods

Plant materials

Seeds of *T. foenum-graecum* were purchased from Aram Seed Company, Seoul, Korea and were kept at 4°C until use. Fifty seeds were sown in each pot having top diameter 116 mm, height 105 mm, bottom diameter 85.5 mm which was filled with vermiculite and placed in a growth chamber under a 16 h photoperiod (300 $\mu\text{mol}/\text{m}^2/\text{s}$) at 25°C.

Chitosan Treatment of Fenugreek Sprouts

A stock solution of chitosan (Sigma-Aldrich) was prepared by addition 10 g chitosan to 1 L of 1% acetic acid (0.1 M) and then the mixture was heated to 40-50°C with continuous stirring, for a period of 24 hrs. Different diluted concentration was prepared according to experimental specification (0.1, 0.5 and 1 mg/ml) from the chitosan stock solution. Same amount of these solutions (0.1, 0.5 and 1 mg/ml) were applied daily up to the time before sampling. All fenugreek sprouts were harvested at 10 days after chitosan treatment. The data were recorded on the following parameters i.e., shoot and root lengths and their fresh weight. The sprouts were frozen in liquid nitrogen and stored at -80°C immediately after collection until used for analysis.

RNA Isolation and cDNA Synthesis

For RNA isolation and cDNA synthesis, samples were grinded using liquid nitrogen and made them into a fine powder and then moved to 1.5 mL micro-centrifuge tubes. Total RNA was separated from the frozen tissues using a Plant Total Mini Kit (Generaid Taiwan) following the protocol described in the manufacturer instructions. After completion of the first step, Trizol was used to homogenize cells; and then chloroform was inserted for phase separation and then added cold isopropanol for RNA precipitation. Purification and total RNA concentration was measured at 260.280 nm (NanoVue Plus Spectrophotometer, GE Healthcare Bio-Science Crop, USA) and RNA integrity was proved on 1% formaldehyde RNA agarose gels. One μg of total

RNA was used for cDNA synthesis. This process was done using a ReverTra Ace- α kit (Toyobo, Japan) for reverse-transcription and then used oligo (dT)₂₀ primer with a 20-fold dilution of the 20- μL resulting cDNA products as template for real time-PCR analysis.

Real-Time PCR

Here in this study qRT-PCR was performed in a BIO-RAD CFX96 Real-time PCR system (Bio-Rad Laboratories, USA). The primers Squalene Synthase (*SQS*), Squalene monooxygenase (*SQLE*) and cycloartenol synthase (*CAS*) were designed using Primer 3 (<http://bioinfo.ut.ee/primer3-0.4.0/>). Sterol-3- β -glucosyl transferase (*STRL*) was arranged from Bioneer, according to transcriptome data of Gujarat Methi-1 variety published (Chaudhary *et al.*, 2015). For housekeeping gene, the 18S gene was used in this study. The PCR products amplified from cDNA were purified by using a Gel Extraction Kit (ELPIS Biotech, Korea) which was used for quantification of the standard and finally the concentration of the products was determined to calculate the number of cDNA copies. The SYBR Green qRT-PCR assay was done in a total volume of 20 μL containing 10 μL of 2 \times Real-Time PCR Smart mix (Biofact Korea), 1 μL (10 pmol/ μL) of each specific primer, 5 μL of template cDNA and 3 μL of D.W. The following conditions as follows: 15 s pre-denaturation at 95°C, followed by 95°C for 20 s; annealing at 55°C for 40 s and 40 cycles of elongation at 72°C for 20 s were maintained. Final extension happened at 72°C for 10 min. The experiments were repeated thrice and the results were represented by means \pm SD.

High Performance Liquid Chromatography Analysis

For the detection of diosgenin, samples were dried in a freeze-dryer at 80°C for about 48 h. An amount of 100 mg dried samples were incubated in 5 mL of 20% H₂SO₄ in 70% isopropanol, under water-bath for 8 h at 80°C. After that, samples were vortexed every 30 min during incubation. Extracts were centrifuged at 12,000 rpm at 4°C for 10 min. After centrifugation, the supernatants were moved to a new 15 mL conical tube and added with 5 mL n-hexane, prior to overtaxing and strong shaking. Next, they were centrifuged at 12,000 rpm at 4°C for 10 min. Only supernatants were moved to new 15 mL conical tubes. For maximum extraction this step was repeated three times. By using a Rotovac evaporator extracts were dried by evaporating the solvent at 40°C in. After that dried crude extracts were solubilized in 1 mL HPLC acetonitrile and filtered using 0.45 μm PTFE hydrophilic syringe filter (Advantec DISMIC-13HP, Toyo Roshi Kaisha, Ltd., Tokyo, Japan) for High Performance Liquid Chromatography (HPLC) analysis. The desired compound diosgenin was isolated on an

Agilent 1100 series HPLC system with a C₁₈ reverse phase column (250×4.6 mm, 5 μm; RStech, Daejeon, Korea) following retention time and detected with a UV detector at 203 nm. Mobile phase was consisted of water as Solvent A and solvent B consisted of acetonitrile. The retention time for diosgenin was 20 min. The initial proportion of the mix was 10% solvent A and 90% acetonitrile. The flow rate of the solvent was maintained at 1.0 ml/min. Diosgenin was detected by matching the retention time and spectral characteristics to those from the single HPLC run of a known diosgenin standard.

Statistical Analysis

All experiments and samples were done thrice for each treatment. The data were analyzed using IBM SPSS Statistic (v24) provides an application-oriented introduction to the statistical component of IBM® SPSS by Tukey's Multiple Range Test., using one-way ANOVA at the 5% significance level.

Results and Discussion

Effects of Chitosan Treatments on Growth, Diosgenin Biosynthetic gene Expression and Diosgenin content in the Sprouts of T. foenum-graecum

The effects of different concentrations of chitosan (0.01, 0.05 and 0.1 mg/mL) on the growth of 10-day old *T. foenum-graecum* sprouts are presented in Fig. 1. The results indicated that, there is no positive response of chitosan in any of the studied growth parameters i.e., shoot and root length, fresh and dry weight. In control treatment without any chitosan solution the highest values for all the parameters were observed. The results indicated that increasing concentrations of chitosan had no significant effect on shoot and root lengths. The fresh weight of sprouts treated with chitosan decreased markedly, but not significantly, compared with that of the control. However, the dry weight of sprouts treated with chitosan was almost similar to that of the control. Growth of root in terms of root length was suppressed greatly than that of shoot length. Here it was observed that the highest root length of 7.9 cm which was observed in control treatment whereas this root length reached to 6.0 cm at the time of application of the highest concentration of chitosan (0.1 mg/ml). On the other hand shoot length did not vary sharply as like as root length. In this case, the highest shoot length of 3.9 cm which was observed in control treatment whereas this shoot length reached to 3.6 cm at the time of application of the highest concentration of chitosan (0.1 mg/ml). In case of fresh weight it was around 1.3 g for the control treatment whereas this value goes below 0.7 g at the time of application of the highest concentration of chitosan (0.1 mg/ml). The variation

in dry weight from control to the highest level of chitosan was almost similar.

The expression of all genes related to the diosgenin biosynthetic pathway in response to different concentration of chitosan enhanced to an increasing level in the expression of all treated sprouts than those of no chitosan (control) treated sprouts (Fig. 2). The highest expression level was detected when treated with 0.05 mg/mL chitosan in most of the genes except *TjSQS*. The expression of *TjSQS* was slightly higher in sprouts treated with 0.01 mg/mL chitosan than in those treated with 0.05 mg/mL chitosan. The expression of *SQS* was higher in 0.01 mg/mL chitosan treated sprouts than in the control, but decreased considerably with an increase in chitosan concentration. The highest expression level was in *TjCAS* at 0.05 mg/mL chitosan exhibiting 3 times higher expressing than that of control. The increasing expression trend for both *TjSQLE* and *TjSTRL* remained up to 0.05 mg/mL chitosan showing 1.7 times more expression in this concentration and then started to decrease the expression level.

Diosgenin content in the *T. foenum-graecum* sprouts did not respond positive to chitosan treatment (Fig. 3). The diosgenin content was higher in control treatment than any one of the chitosan treatment. It is notated that chitosan treatment suppressed the accumulation of diosgenin content.

It is observable that with no chitosan treatment showed the positive response for the growth parameters like shoot and root length and their fresh and dry weight. The highest values for all the parameters were observed in control treatment without any chitosan treatment But due to the treatment of chitosan the diosgenin biosynthetic genes i.e., *TjSQS*, *TjSQLE*, *TjCAS* and *TjSTRL* were upregulated. In relation with our studied some previous research findings have shown that chitosan enhanced to accumulate higher amount of secondary metabolites. In a previous study reported that chitosan enhanced to accumulate sorgoleone in sorghum roots (Uddin *et al.*, 2010). Chitosan belongs to a family of biopolymers naturally and industrially obtained by *N*-deacetylation of chitin, which is almost never complete (Ferri and Tassoni, 2011). Chitosan treatment increased the total weight by 12.9% and the germination rate by 16% compared to those of the control in sunflower sprouts (Cho *et al.*, 2008). Further, with an increase in chitosan concentration from 2 to 8 mg/mL, the phenolic acid accumulation increased, such as trans-cinnamic acid, benzoic acid, caffeic acid, ferulic acid and sinapic acid (Reddy *et al.*, 1999). No correlation was found between gene expression and chitosan concentration in fenugreek sprouts. These results indicated that, although chitosan concentration and gene expression varied in sprouts, chitosan treatment was not a viable option for increasing diosgenin content in fenugreek sprouts.

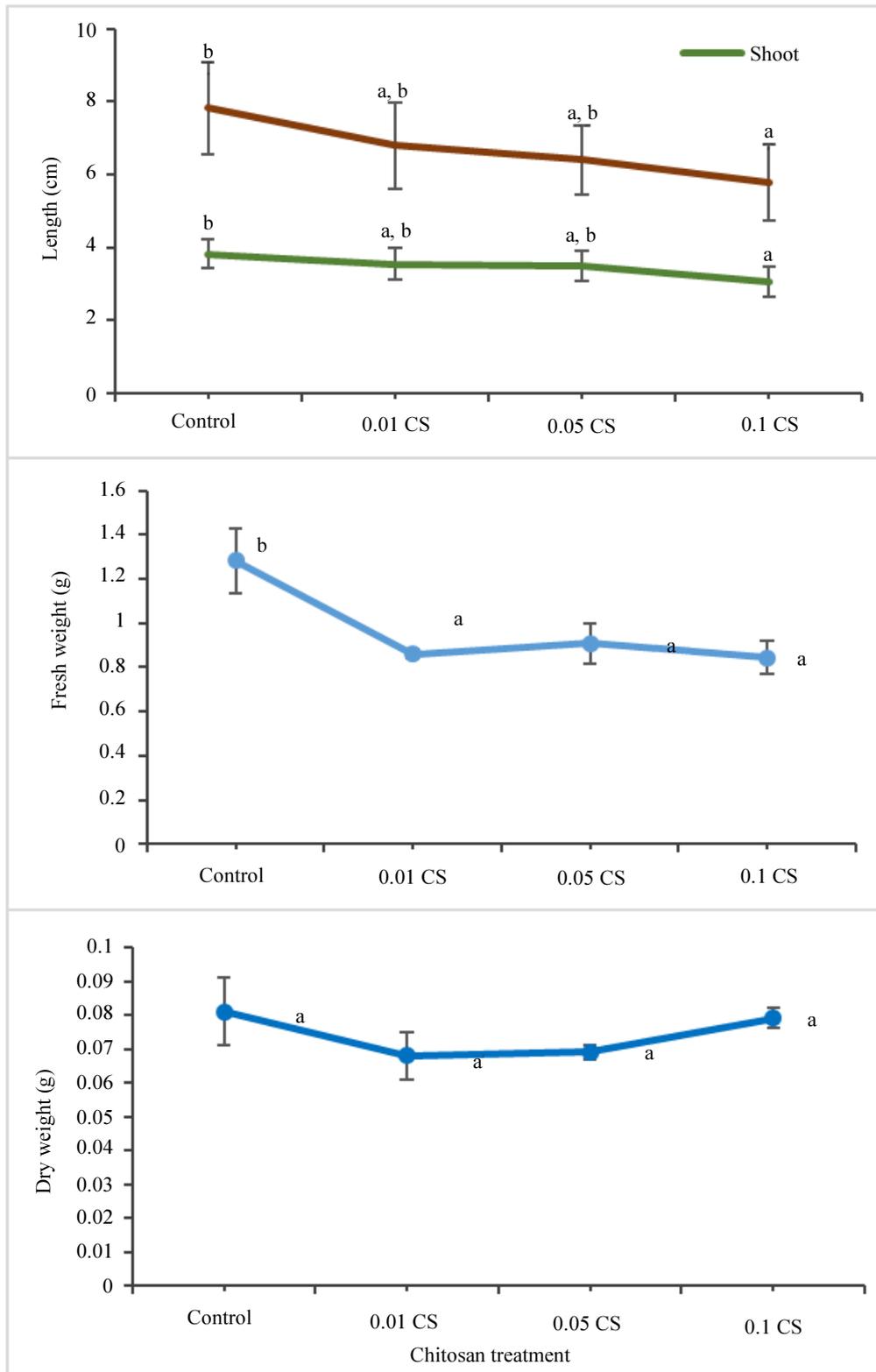


Fig. 1: Plant growth of fenugreek sprouts treated with different concentrations of chitosan. The final chitosan concentrations were 0.1, 0.5 and 1 mg/mL ($n = 3$). $R^2 = 0.9992$ and the bars with different letters are significantly different from each other at $p \leq 0.05$ according to Tukey's Multiple Range Test

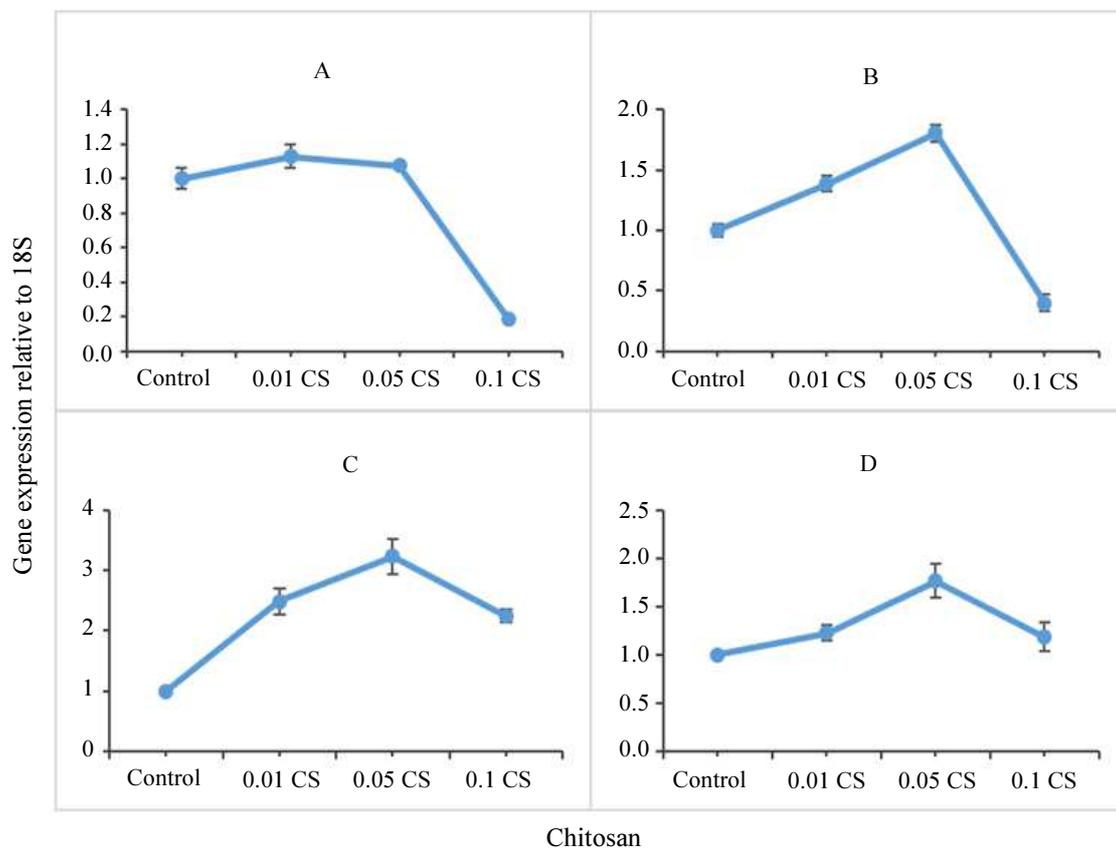


Fig. 2: Expression of diosgenin biosynthetic genes in fenugreek sprouts treated with different chitosan concentrations. The final chitosan concentrations were 0.1, 0.5 and 1 mg/mL ($n = 3$). The graph of (A), *TfSQS*: *Trigonella foenum graecum* squalene synthase; (B), *TfSQLE*: *Trigonella foenum graecum* squalene monoxygenase; (C), *TfCAS* *Trigonella foenum graecum* cycloartenol synthase; and (D), *TfSTRL*: *Trigonella foenum graecum* sterol-3- β -glucosyl transferase

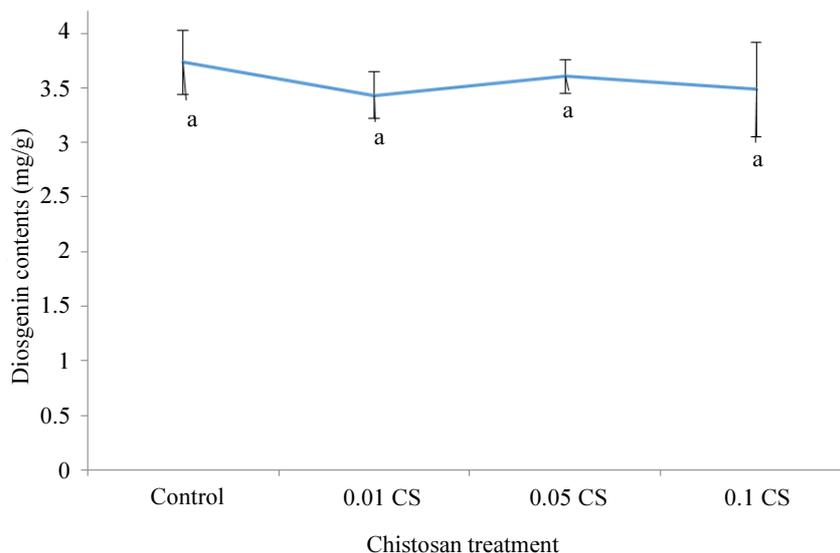


Fig. 3: Diosgenin content (mg/g DW) in chitosan-treated sprouts. The final chitosan concentrations were 0.1, 0.5 and 1 mg/mL ($n = 3$). $R^2 = 0.9992$ and the bars with different letters are significantly different from each other at $p \leq 0.05$ according to Tukey's Multiple Range Test

Conclusion

Diosgenin is the principal precursor compound in the manufacture of several synthetic steroidal drugs. It is acted as a promising bioactive biomolecule exhibiting various biological properties, like hypolipidemic, hypoglycaemic, antioxidant, antiinflammatory and antiproliferative activities. Sprouts of *T. foenum-graecum* treated with elicitor like chitosan responded negatively both for shoot and root growth, but these treatments enhanced the upregulation of diosgenin biosynthetic genes i.e., *TjSQS*, *TjSQLE*, *TjCAS* and *TjSTRL*. Variations in diosgenin levels remained almost similar in the chitosan treatment compared to control. Though chitosan did not response well for growth and accumulation of diosgenin in the sprouts of *T. foenum-graecum* but showed upregulation trends of all diosgenin biosynthetic genes. These information might be helpful for future study in this arena.

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Author's Contributions

Do Yeon Kwon: Performed the experiments, analyzed the data and prepare the manuscript.

Jaе Kwang Kim: Performed the experiments, analyzed the data and prepare the manuscript.

Sang Un Park: Designed the experiments, coordinated the implementation of research work.

Ethics

This manuscript has not been published or presented elsewhere in part or in entirely and is not under consideration by another journal. All the authors have approved the manuscript and agree with submission to your esteemed journal. There are no conflicts of interest to declare.

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