Article

Virus-Induced Gene Silencing of *SIWRKY79* Attenuates Salt Tolerance in Tomato Plants

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Abstract: Previous studies have shown that WRKY transcription factors play important roles in abiotic stress responses. Thus, virus-induced gene silencing (VIGS) was used to identify the function of *SIWRKY79* in the salt tolerance of tomato plants by downregulating the expression of the *SIWRKY79* gene. Under the same salt treatment conditions, the *SIWRKY79*-silenced plants showed faster stem wilting and more severe leaf shrinkage than the control plants, and the bending degree of the stem of the *SIWRKY79*-silenced plants was also greater than that of the control plants. Physiological analyses showed that considerably higher levels of hydrogen peroxide (H₂O₂), superoxide anion (O₂^{•−}), and abscisic acid (ABA) accumulated in the leaves of the *SIWRKY79*-silenced plants than in those of the controls after salt treatment. Taken together, our results suggested that *SIWRKY79* plays a positive regulatory role in salt tolerance in tomato plants.

Keywords: *SIWRKY79*; salt resistance; VIGS; tomato

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1. Introduction

Salt stress is a major abiotic stress that threatens crop production and has significantly affected agriculture [1,2]. In recent years, significantly increased soil salinization levels have seriously affected tomato production [3]. Therefore, it is necessary to identify salt tolerance-related genes, verify their functions, and cultivate salt-tolerant tomato varieties.

The WRKY transcription factor family is one of the largest transcription factor families in plants; these proteins are involved in regulating plant signaling networks [4], and their structures include zinc finger domains [5,6]. The WRKY signaling network responds to both biotic and abiotic stresses [7]. Most studies to date have examined WRKY transcription factors involved in biotic stress, such as disease resistance and immune response mechanisms [8]. However, in recent years, many studies have also reported the roles of WRKY transcription factors in abiotic stress. For instance, microarray analysis in *Arabidopsis* revealed that 18 AtWRKY genes were involved in salt tolerance regulation [9]. The regulatory function of WRKY25 was identified by analysis of the WRKY25 mutant and transgenic *Arabidopsis thaliana* plants overexpressing WRKY25, and the results showed that WRKY25 played a key role in heat resistance [10]. Transcriptome analysis of rice seedlings under water deficit revealed that several WRKY genes were upregulated under drought conditions, and these genes were involved in the regulation of water deficit stress [11].

Virus-induced gene silencing (VIGS) is an effective method of studying gene function [12,13]; this technique can rapidly decrease gene function by inhibiting gene expression. Transcriptome data revealed that the *SIWRKY79* gene is differentially expressed under salt stress, and experiments were performed to silence this gene. The purpose of this study was to identify the function of the *SIWRKY79* gene under salt stress. In addition, the

regulatory relationships of *SIWRKY79* with other genes involved in tomato salt tolerance were detected by quantitative real-time polymerase chain reaction (qRT-PCR) analysis.

2. Materials and Methods

2.1. *SIWRKY79* Gene Sequence Analysis

Two reported genes from Group I, Group II (IIa, IIb, IIc, IId, and IIe), and Group III of the tomato WRKY family were selected, separately. The amino acid sequences were downloaded from the SGN database (<https://solgenomics.net/>). Sequence alignment was performed with the DNAMAN 8 software, and homology analysis was performed using the MEGA X software.

2.2. Plant Growth and Treatment

Tomato (*Solanum lycopersicum* L. cv. Moneymaker) seedlings were grown in a growth chamber at Dongbei Agricultural University with a 16-h light (24 °C)/8-h dark (20 °C) photoperiod, and the light intensity was 40,000 lx [2,14]. Eighteen four-leaf stage tomato plants were used. Of these plants, 9 plants were subjected to gene silencing treatment, and 9 plants were used as controls. After the plants were infected with the bacterial solution, the infected plants and the control plants were placed in a light incubator for 3 weeks until photobleaching occurred. At four weeks after treatment, silenced plants and control plants were washed with sterile water, transferred to an Erlenmeyer flask containing 1/2-strength Hoagland nutrient solution for two days, and then transferred to a 200 mM sodium chloride solution for salt stress treatment. The changes in plant phenotype were observed at 0, 4, and 8 h. Samples were taken at each time point for subsequent analysis. Three biological replicates were included.

2.3. Amplification of the Target Fragment

The mRNA sequence of the *SIWRKY79* gene and Primer 5 software were used to design the following primers containing *EcoRI* and *BamHI* digestion sites: 79-F: 5'-CGGAATTCCGTCACCGCGTTCCGGTTTATT-3' and 79-R: 5'-CGGGATCCCCGCCGGCCA-AGGATGGTTATGT-3'. The primers were analyzed by PCR and agarose gel electrophoresis, and the correct size bands were removed from the gel and purified using a PCR purification kit (Takara) [15]. The purified products were subsequently cloned into a TRV2 vector (Takara) for sequencing.

2.4. Construction and Infection of the VIGS Vector

The TRV2 vector and target fragment were double digested with *EcoRI* and *BamHI* and then verified by agarose gel electrophoresis. The bands were cut from the gel and recovered using a gel recovery kit. The recovered vector and the target fragment were then ligated with T4 ligase. The ligated vector was transferred into *E. coli* DH5 α competent cells, and a single colony was selected and grown on liquid Luria–Bertani broth (LB) medium containing 50 μ g/mL kanamycin. The cultured bacterial solution was verified by sequencing, and the constructed vector was then extracted with a plasmid extraction kit. TRV1, TRV2-00, TRV2-*PDS*, and TRV2-*SIWRKY79* were transferred into *Agrobacterium* GV3101 and grown on LB medium containing 50 μ g/mL kanamycin and 50 μ g/mL rifampicin for 24–48 h. A single colony was selected and incubated in a liquid medium for 24–48 h until the optical density at 600 nm wavelength (OD_{600}) = 0.5. Then, the bacteria were centrifuged, and cells were collected and resuspended in IM buffer. The TRV2-00, TRV2-*PDS*, and TRV2-*SIWRKY79* bacterial solutions were mixed individually with the TRV1 bacterial solution at a ratio of 1:1 to obtain the bacterial infection solutions. When the seedlings reached the four-leaf stage, they were used in the virus-induced gene silencing (VIGS) experiment. The bacterial solution was infiltrated into the plant leaves and tender stems with a 1-mL syringe.

2.5. Tissue-Specific Expression Analysis

Roots, stems, and leaves were extracted from the plants at three time points during the stress treatment: 0, 4, and 8 h. The TRIzol method was used to extract RNA from the tomato roots, stems, and leaves, and gene expression levels were analyzed by qRT-PCR as reported in previous studies [15]. EF α 1 was used as a reference gene for normalization [15], and the relevant data were analyzed using the $2^{-\Delta\Delta CT}$ method. The TBtools software was used to analyze the expression of the WRKY family genes in tomato tissues and to construct a heat map [16].

2.6. 3,3'-Diaminobenzidine (DAB) and Nitro Blue Tetrazolium e (NBT) Tissue Staining

The leaves were stained with DAB and NBT staining solutions to determine the hydrogen peroxide (H₂O₂) and superoxide anion (O₂^{•−}) contents. DAB forms a red-brown precipitate when exposed to H₂O₂, and NBT and O₂^{•−} react to produce a dark blue precipitate [17].

2.7. Abscisic Acid (ABA) Assays

Leaf tissues were homogenized and extracted with 80% methanol solution. The extraction solution was then collected by centrifugation and extracted again. The extract was filtered, evaporated to increase its concentration, and then adjusted to pH 8.0 with 0.2 mol/L Na₂HPO₄. The concentrate was extracted twice with a mixture of petroleum ether and ethyl acetate, adjusted to pH 2.8 with 0.2 mol/L citric acid, and then extracted three times with ethyl acetate. The extract was condensed and evaporated, dissolved in the mobile phase, and then filtered into a concentration bottle through a membrane.

3. Results

3.1. SIWRKY79 Gene Sequence Analysis

The protein sequence of SIWRKY79 was compared with those of SIWRKY8, SIWRKY11, SIWRKY17, SIWRKY23, SIWRKY32, SIWRKY33, SIWRKY37, SIWRKY39, SIWRKY45, SIWRKY57, SIWRKY74, SIWRKY80, and SIWRKY81 (Figure 1), and a phylogenetic tree was constructed (Figure 2). SIWRKY79 exhibited the highest homology with SIWRKY37 in the same group.

SIWRKY8	KVYSFTFIQQVELITYLHFNELVEKTIISFSYSEEMSPTNS	159
SIWRKY11	AVKVFVEKYQSKAITLLFTKPKVKKSIGCEAVFVASSTIS	144
SIWRKY17	GNISINNKEADQATEATMPKARVSVRARSEAFMITDGCQW	359
SIWRKY23	TNAKQQLKAKKTIVSQKQKEFRFAFMKSEVLFLEDGYRW	175
SIWRKY32	RRSAAVNCSTGEVVKQSSDAQVLAIVFVIKRETRDGYNW	208
SIWRKY33	EVAFIQSFSQENMSNNEAEVHYCQPSQYVRECKAEDGYNW	202
SIWRKY37	QFLASKNPKMAQKVVMIVQMEAKSLKQKNEGFEF.DCWSW	58
SIWRKY39	LELSSTKKPREEHIKTKTSRVYMRTEFSDISLIVKDGQW	177
SIWRKY45	FEFASPKRPRE..ITNVSTVCIKTNRSDQTSVVKDGYNW	135
SIWRKY57	RPFSLTASKVKKKQCKRIRQFRFAFMKSEVLFLEDGYRW	183
SIWRKY74	KTMRNNGGLDVSQCNFTKRARVSVFVRCIAFTMNDGCQW	284
SIWRKY79	SPKRSRRSIEKRVVSFVIEIEGSKMKGEISMFPDLSAW	82
SIWRKY80	GSELKKKKKEKQDRFGCYKRRKTSGSWMRESATMNDGCAW	121
SIWRKY81	EDYSSGSCKSSLRFGCYKRRKTSSESLIKESSLIVDLGHAW	115
Consensus		

Figure 1. Cont.

SIWRKY8	FNISSLTIGETESKQSSSSNSAFQ..MTN.ISSQVNSACK	196
SIWRKY11	...SSFMSTITGEGSVSNGKVFS..SMS.IPFRPFVSSGK	178
SIWRKY17	RKYGQKMAKCNFCFRAYYRCTMA..AGCFVRKQVCFCAED	397
SIWRKY23	RKYGQKAVKNSFFFRNYRCTN...ATCNVKKRVERCFSD	212
SIWRKY32	RKYGQKQVKSFPQGSFSYYRCTHS..ECC..AKKIECSHT	244
SIWRKY33	RKYGQKQVKSSENERFSYYKCTF...ENCPTKKKVEFNLLG	239
SIWRKY37	RKYGQKFIKCSFYFRGYRCSFS..KGCSAKKQVERCSKD	96
SIWRKY39	RKYGQKVIRLNFSEFRAYFKCSFA..FTCFVKKKVCPSVED	215
SIWRKY45	RKYGQKVIRLNFSEFRAYFKCSFA..FTCFVKKKVCPSIED	173
SIWRKY57	RKYGQKAVKNSFFFRNYRCTN...TKCTVKKRVERSED	220
SIWRKY74	RKYGQKIAKCNFCFRAYYRCTVA..ENCQVRKQVCFCAED	322
SIWRKY79	RKYGQKFIKCSFYFRGYRCSFS..KGCFARKQVERSRAD	120
SIWRKY80	RKYGQKKILNSKYFRCTHKYDQECRATKQVCIQEN	161
SIWRKY81	RKYGQKQILNSTYFRHYFRCTHKYDQCKQASKQVCIQEN	155
Consensus		
SIWRKY8	EPISSS.SIKRKCSISEN.....AVSGKC	219
SIWRKY11	EPIAGK.FCR.DHEISL.....EFSGRT	199
SIWRKY17	RTILIT.TYEGTHNHPLFFAAIAMASTISSAAKMILSGSM	436
SIWRKY23	ESIVVT.TYEGKHTHPSF.....MNT	232
SIWRKY32	NRVMEI.IYFTQHNDFFFRVNCERESKSALLSFTNGKS	283
SIWRKY33	.HITEI.VYKGSNNHFKFCSTR..RSSQSIQNLAYSND	275
SIWRKY37	ASIFII.TYTSSNNHPCF.....NLEKLS	119
SIWRKY39	QSILVA.TYEGEHNHPSK.....ITAGFV	238
SIWRKY45	ESILVG.VYEGEHNHP.....	188
SIWRKY57	SSIVIT.TYEGQHCHH.....TVGF	240
SIWRKY74	MSILIT.TYEGTHNHTIFLSATAMASTISAAASMLSGSS	361
SIWRKY79	ENMLIV.TYSCEHNHPWEAS.....FNNQHNR	147
SIWRKY80	FIIMYHTTYEGNHTCN.....ET	179
SIWRKY81	EQRFET.TYEGHHTICKAFF.....FVSQ	177

Figure 1. Alignment of the *SIWRKY79* protein sequence with those of *SIWRKY8*, *SIWRKY11*, *SIWRKY17*, *SIWRKY23*, *SIWRKY32*, *SIWRKY33*, *SIWRKY37*, *SIWRKY39*, *SIWRKY45*, *SIWRKY57*, *SIWRKY74*, *SIWRKY80*, and *SIWRKY81*. Pink means higher similarity, light blue means lower similarity.

Figure 2. Phylogenetic tree of *SIWRKY79*, *SIWRKY8*, *SIWRKY11*, *SIWRKY17*, *SIWRKY23*, *SIWRKY32*, *SIWRKY33*, *SIWRKY37*, *SIWRKY39*, *SIWRKY45*, *SIWRKY57*, *SIWRKY74*, *SIWRKY80*, and *SIWRKY81*.

3.2. Plant Phenotype Observation under Salt Stress Treatment

After 4 h of salt stress, the TRV2-*SIWRKY79* plants showed more wilting than the TRV2-00 plants. The leaves were slightly curled, and the stems became slightly soft (Figure 3). At 8 h of salt treatment, the TRV2-*SIWRKY79* plants were completely wilted, and the stems had softened completely (Figure 3). In addition, the leaves showed severe curling. The TRV2-00 plants had completely wilted at 24 h. In short, the wilting degree of the gene-silenced plants was significantly greater than that of the control plants. This phenomenon indicated that the salt tolerance of the plants was reduced after the downregulation of the *SIWRKY79* gene.

Figure 3. Phenotypic changes in TRV2-00 and TRV2-*SIWRKY79* tomato plants during the salt stress treatment.

3.3. Analysis of *SIWRKY79* Gene Expression in Different Tissues after Salt Stress Treatment

We compared the expression levels of *SIWRKY79* in three plant tissues: root, stem, and leaf. No significant differences in *SIWRKY79* expression levels of untreated plants were noted during different time periods or in different tissues (Figure 4D). In the treated plants, *SIWRKY79* expression in stems at 4 h was significantly higher than that at 0 h and 8 h, while *SIWRKY79* expression in leaves at 8 h was significantly higher than that at 0 h and 4 h (Figure 4E). In addition, the TRV2-*SIWRKY79* plants had lower *SIWRKY79* expression levels than the TRV2-00 plants at each time point (Figure 4A–C). Overall, after gene silencing, *SIWRKY79* expression levels in the roots decreased, indicating that the *SIWRKY79* gene participates in the regulation of salt tolerance in tomato plants.

Figure 4. Comparison of *SIWRKY79* gene expression levels in roots, stems, and leaves at each time point. (A), Comparison of *SIWRKY79* gene expression levels in roots; (B), comparison of *SIWRKY79* gene expression levels in stems; (C), comparison of *SIWRKY79* gene expression levels in leaves; (D), comparison of *SIWRKY79* gene expression levels in the roots, stems, and leaves of TRV2-00 plants at different time points; (E), comparison of *SIWRKY79* gene expression levels in the roots, stems and leaves of TRV2-*SIWRKY79* plants at different time points; (F), changes in ABA contents in TRV2-00 and TRV2-*SIWRKY79* leaves after 0, 4, and 8 h of treatment. The data presented are the means $\pm$ standard deviations (SDs) of three independent experiments, and the different letters above the columns indicate significant differences at the $p < 0.05$ level. *: $0.01 < p < 0.05$, **: $p < 0.01$, and n.s.: $p > 0.05$.

We selected all of the genes of the WRKY transcription factor family and used the TBtools software to analyze the differentially expressed genes in various tissues (Figure 5). The analysis showed that the salt tolerance-related genes *SIWRKY13*, *SIWRKY24*, *SIWRKY31*, *SIWRKY50*, *SIWRKY62*, *SIWRKY63*, and *SIWRKY79* were highly expressed in the roots (Figure 5).

Figure 5. Heat map of differential expression of the WRKY transcription factor family genes in different tissues.

3.4. Gene Silencing Significantly Increased Endogenous ABA Content

During salt treatment, the endogenous hormone ABA gradually accumulated over time. ABA levels in TRV2-*SIWRKY79* plants were greater than those of TRV2-00 plants at each time point. At 0, 4 and 8 h, the ABA content in TRV2-*SIWRKY79* plants was 36%, 23%, and 230% greater than that in TRV2-00 plants, respectively. In addition, the accumulation rate of ABA in the gene-silenced plants was higher, especially from 4–8 h. At this time point, ABA accumulation in the TRV2-*SIWRKY79* plants increased by 299%, while that in the TRV2-00 plants increased by only 48% (Figure 4F). Overall, the ABA level was higher in TRV2-*SIWRKY79* plants but only significantly higher at 8 h. These results indicate that the *SIWRKY79* gene may negatively regulate the ABA signaling pathway.

3.5. Salt Treatment Led to Greater ROS Accumulation in TRV2-*SIWRKY79* Plants Than in TRV2-00 Plants

After DAB and NBT staining, the red-brown areas were mainly concentrated at the tip and edge of the leaves (Figure 6A–F), while the dark blue areas were mainly concentrated at the leaf base, in the main vein, and in its vicinity (Figure 6G–L). The stained areas gradually increased with increasing treatment time, and the TRV2-*SIWRKY79* plant samples were darker in color than were those of the TRV2-00 plant samples at each time point. These results showed significantly higher ROS accumulation in the TRV2-*SIWRKY79* plants compared with the TRV2-00 plants.

Figure 6. After salt treatment, H_2O_2 and O_2^- accumulation in leaves increased with increasing time. (A–F), H_2O_2 accumulation indicated by DAB staining; (G–L), O_2^- accumulation indicated by NBT staining.

3.6. *SIWRKY79* Gene Silencing Reduced the Expression of Related Salt Tolerance-Related Genes

The tomato *SINAM1*, *SINAC1*, *SIWRKY23*, and *SIRD22* genes were compared, and several salt tolerance-related genes were expressed in plants. The results showed that the expression levels of these genes in TRV2-*SIWRKY79* plants were lower than those in

TRV2-00 plants (Figure 7), and it was hypothesized that *SIWRKY79* silencing resulted in a significant decrease in the expression levels of these genes. These results indicate that *SINAM1*, *SINAC1*, *SIWRKY23*, and *SIRD22* interact with *SIWRKY79* and may be located downstream of the *SIWRKY79* gene signaling pathway. Therefore, we proposed a model of *SIWRKY79* gene salt tolerance regulation (Figure 8). In this model, external salt stress stimulates *SIWRKY79* gene expression and then induces the expression of the downstream *SINAM1*, *SINAC1*, *SIWRKY23*, and *SIRD22* genes to resist salt stress.

Figure 7. Comparison of *SINAM1*, *SINAC1*, *SIWRKY23*, and *SIRD22* expression in TRV2-00 and TRV2-*SIWRKY79* cells. The data presented are the means $\pm$ SDs of three independent experiments, and the different symbols above the columns indicate significant differences at the $p < 0.05$ level. **: $p < 0.01$.

Figure 8. A salt tolerance signaling pathway may be induced by the *SIWRKY79* gene.

4. Discussion

Plants are subjected to numerous abiotic stresses, such as flooding, low temperature, and salinity, during the growth process, and these stresses can cause great losses in production [18]. Therefore, the study of abiotic stress is particularly important for agriculture. At present, an increasing number of researchers are focusing on functional analyses of WRKY transcription factors in many model plants under abiotic stresses, such as flooding and drought. A series of changes, such as stomatal closure and the expression and induction of related genes, occur in plants in response to abiotic stress [19]. Substantial evidence indicates that WRKY transcription factors participate in [20] and may play very important roles [21] in abiotic stress responses. The *SIWRKY79* gene examined in this study belongs to the WRKY family. By silencing the *SIWRKY79* gene, we found that it was involved in regulating salt tolerance in tomato plants and had a positive effect on resistance to salt stress. These results are consistent with previous research results and suggest that

SIWRKY79 participates in stress resistance [15]. However, *SIWRKY79* expression levels in the stem of gene-silenced plants was significantly higher than that in control plants at 4 h. We hypothesize that at this time point, the *SIWRKY79* gene was still weakly expressed in the stem. This weak expression is a limitation of gene silencing, but the overall gene expression trend decreased, indicating that the *SIWRKY79* gene was silenced. The expression of the salt tolerance-related genes *SINAM1*, *SINAC1*, *SIWRKY23*, and *SIRD22* in gene-silenced plants was reduced; therefore, we hypothesize that *SIWRKY79* regulates upstream *SINAM1*, *SINAC1*, *SIWRKY23*, and *SIRD22* expression and jointly regulates the salt tolerance network mechanism.

ROS are constantly produced in the cells of plants, and their levels are increased in a variety of adverse conditions [17]. These adverse conditions include both biotic and abiotic stresses, such as environmental stress, pathogens, and damage. Under these stress conditions, ROS are produced in large quantities in the photosynthetic apparatus during photorespiration and mitochondrial respiration [22]. This overproduction causes ROS to attack intracellular substances, causing cellular damage [15,23]. ROS are associated with many abiotic stresses, as has been shown in *Arabidopsis thaliana* [18]. In the stress response, ROS are a sign of successful recognition of infection and activation of plant defense, and O_2^{2-} and H_2O_2 are the most important and stable ROS products [24]. In this study, the ROS content in the plants after gene silencing was apparently greater than that in the unsilenced plants. The H_2O_2 produced was mainly concentrated in the leaf edges, while O_2^{2-} was mainly concentrated in and around the leaf veins. This ROS overproduction severely damaged the plants, and the plants gradually withered. This phenomenon indicated that the *SIWRKY79* gene can positively regulate plant salt stress resistance.

The endogenous hormone ABA is involved in regulating adaptation to environmental stresses [25]. It not only regulates the stomatal opening and the growth and development of plants but also coordinates various stress signal transduction pathways in plants during abiotic stress [26]. Studies have shown that the WRKY family is involved in the ABA signal transduction pathway [21,27,28]. In addition, the *LTWRKY21* gene, previously isolated from the xerophyte *Larrea tridentata* (creosote bush), has been shown to activate ABA signaling [29], while *WRKY40/WRKY18* and *WRKY60* are negative regulators of ABA signaling during seed germination and growth in *Arabidopsis thaliana* [30]. In this study, the ABA content increased gradually with increasing stress duration, and the amount of ABA accumulation in the *SIWRKY79*-silenced plants was apparently greater than that in the unsilenced plants. These results indicate that *SIWRKY79* is induced by ABA and responds to the ABA signaling pathway. The silencing of this gene significantly reduces the salt tolerance of tomato plants. We discovered for the first time that the *SIWRKY79* gene in the WRKY family is involved in salt tolerance in tomatoes. These results were consistent with those of a previous study [31].

Overall, the *SIWRKY79* gene was silenced in tomato plants by VIGS, and the *SIWRKY79* gene expression response was the most obvious in roots. In addition, *SIWRKY79* inhibited ROS and ABA production and improved plant resistance. These results showed that *SIWRKY79* may mediate the ABA signaling pathway and salt tolerance in tomato plants. Therefore, we hypothesize that the *SIWRKY79* gene plays an active regulatory role in salt tolerance in tomato plants.

Author Contributions: All the authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Y.H. and J.J. The first draft of the manuscript was written by Y.H. and J.J. and all authors commented on previous versions of the manuscript. All the authors read and approved the final manuscript. Conceptualization, T.Z., X.X. and J.L.; Methodology, Y.H., J.J., X.Z. and H.Y.; Formal analysis, Y.H., J.J., X.Z., Y.T. and D.S.; Writing—original draft, Y.H. and J.J.; Writing—review & editing, Y.H. and J.J.; Supervision, H.Y. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: We declare that we have no conflict of interest.

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