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ORIGINAL ARTICLE

Ectopic expression of nucleolar DEAD-Box RNA helicase *OsTOGR1* confers improved heat stress tolerance in transgenic Chinese cabbage

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Abstract

Key message **The DEAD-Box RNA helicase Os*TOGR1* positively regulates heat stress tolerance in Chinese cabbage.**

Abstract Non-heading Chinese cabbage (*Brassica rapa* L. ssp. *chinensis*) is primarily cultivated vegetable crop in Asian countries. Heat stress is one of the major threats for its growth and yield. Numerous regulatory genes in various crops have shown to contribute thermotolerance. Among them, Thermotolerant growth required 1 (*TOGR1*) is an important DEAD-box RNA helicase. To examine whether its role is conserved in other crops, we constructed *pCAMBIA1300-pHSP:OsTOGR1* expression vector driven by the rice small heat shock protein promoter (p*HSP17.9*) and successfully produced transgenic non-heading Chinese cabbage plants expressing *OsTOGR1* gene via *Agrobacterium*-mediated vacuum infiltration transformation. In total, we generated three independent transgenic cabbage lines expressing *TOGR1* gene. Expression and integration of *TOGR1* was confirmed by PCR, RT-PCR and qPCR in T_1 and T_2 generations. The relative leaf electrical conductivity of transgenic seedlings was reduced subjected to high temperature (38 °C) compared to heat shock treatment (46 °C). In addition, hypocotyl length of transgenic seedlings increased compared to wild-type plants under high temperature and heat shock treatment. Furthermore, the transgenic plants exhibited higher chlorophyll content than wild-type plants under high temperature and heat shock treatment. The transgenic seeds displayed better germination under heat shock treatment. Tested heat stress-responsive genes were also up-regulated in the transgenic plants subjected to high temperature or heat shock treatment. To the best of our knowledge, this is the first report on describing the role of DAED-Box RNA helicases in improving heat stress tolerance of transgenic plants.

Keywords *TOGR1* · thermotolerant growth required 1 · *HSP* · heat shock protein · Non-heading Chinese cabbage

Introduction

The sixth report of the Intergovernmental Panel on Climate Change mission aspires to abate the rise in earth's temperature (Xu et al. 2018). High temperature is considered to be a major environmental factor limiting crop growth and productivity (Zhang et al. 2019; Driedonks et. al. 2016; Gangadhar et al. 2016). Heat stress, 5 °C above the optimal growth temperature of plants induces an array of cellular and molecular changes to withstand high-temperature conditions (Kaushal et al. 2016; Bita and Gerats 2013). Those changes attributed to reactive oxygen species (ROS), hormone signaling and heat shock protein-dependent pathways (Suzuki and Katano 2018). High temperature severely affects the vegetable crops yield and nutritional quality (Scheelbeek et al. 2018). Development of transgenic crops with improved thermotolerance is one of the most critical traits for sustainable food production in recent days (Zhang et al. 2019; Wang et al. 2019; Jiang et al. 2018; Li et al. 2015). The adverse effects imposed by heat stress in plants can be mitigated by exogenous application of PGRs, microbes, suitable mineral nutrition, by screening heat-tolerant cultivars and by genetic engineering approaches (Ali et al. 2019; Lavania et al. 2015; Fragkostefanakis et al. 2015). Genetic engineering approaches were successfully employed to enhance

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thermotolerance in various plants (Huo et al. 2020; Chen and Qiu 2020; Zhang et al. 2016). However, genetic improvement in crops for high-temperature tolerance is hampered by lack of gene resources that impart high tolerance against temperature stress.

RNA helicases are ubiquitous in nature and participated in RNA metabolism of both prokaryotic and eukaryotic organisms (Jankowsky 2011). The DEAD-Box RNA helicases are the largest RNA helicase family that contains helicase core domain of nine conserved motifs (Huang et al. 2016). The DEAD-Box helicases contain amino acids Asp-Glu-Ala-Asp (DEAD) and have been associated with an array of biological processes in plants especially in abiotic stress adaptation (Nidumukkala et al. 2019; Liu et al. 2018; Baruah et al. 2017). A number of studies have been carried out to express DEAD-Box RNA helicases for enhancing abiotic stress tolerance in transgenic plants (Nguyen et al. 2018; Shivakumara et al. 2017; Singha et al. 2017; Tuteja et al. 2014; Sanan-Mishra et al. 2005). Recently, our group identified a nucleolar located DEAD-Box RNA helicase by map-based cloning of rice, namely 'Thermotolerant growth required 1' (*TOGR1*). Normal plant growth at high temperature is controlled by *TOGR1* recruited to the smaller sub-unit (SSU) of ribosome in the nucleolus to ease an effective pre-rRNA processing essential for normal cell division (Wang et al. 2016). The *TOGR1* shown to be intricated in thermotolerance by associating with preRNA processosome and maintaining normal ribosomal RNA levels at high temperatures *via* elevating helicase activity (Wang et al. 2016). Till now, none of the reports have been published to describe the role of DEAD-Box RNA helicases in thermotolerance of plants. Moreover, over- expression of *TOGR1* in rice significantly improved the rice growth and yield under hot conditions (Wang et al. 2016). In order to know, whether the function of this monocot gene is conserved in dicot plants, we generated transgenic Chinese cabbage plants by heterologous expression of *TOGR1* under the control of small 17.9 *HSP* gene promoter.

Non-heading Chinese cabbage (*Brassica rapa* L. ssp. *chinensis*) is one of the most important vegetables in China and other eastern Asian countries. Being native plant of China, it has a long cultivation history across the country (Wang et al. 2016; Song et al. 2014). Especially, the leaves are supplemented with crucial mineral elements, crude fiber, as well as vitamin supplements. Heat stress affects the reproductive phase of *B. rapa* plants (Jiang et al. 2018; Yu et al. 2012). Reports also suggested that high temperature has greater influence on yield and seed quality of *B. rapa*, more adversely than its vegetative growth (Angadi et al. 2000). Hence, developing thermotolerant cabbage plants is essential for vegetable production (Jiang et al. 2018). Genetic transformation is an important tool for producing thermotolerant transgenics for sustainable food production. Genetic transformation of Chinese cabbage is quite challenging as it is recalcitrant to tissue culture approach (Zhang et al. 2000; Narasimhulu and Chopra 1988). Genetic transformation efficiency in *B.rapa* plants is mostly genotype dependent. Alternatively, *inplanta* plant transformation methods, such as vacuum infiltration of *Agrobacterium*, are the better approaches for genetic transformation of Chinese cabbage plants (Hu et al. 2019; Zhang et al. 2011; Xu et al. 2008; Qing et al. 2000).

To determine whether ectopic expression of *TOGR1* improves thermotolerance, *TOGR1* was fused to a small heat shock protein promoter sequence and introduced into cabbage genome. The role of nine members of cytosolic class I small heat shock proteins (sHSP-CI) in rice was characterized under heat stress. Among them, *Oshsp17.9* was strongly induced by heat stress (Guan et al. 2004). Therefore, we employed the promoter of *Oshsp17.9* to drive the expression of *TOGR1* in this study. The vector *pCAMBIA1300* was used to clone the *TOGR1* and *hsp* promoter, where *NPTII* selection marker is under the control of 35S promoter. To the best of our knowledge, this is the first report on generating thermotolerant Chinese cabbage plants via *Agrobacterium*-mediated vacuum infiltration method. In this study, transgenic Chinese cabbage plants expressing rice *TOGR1* gene were generated. We confirmed that *TOGR1* gene was successfully integrated to the cabbage genome and expressed in the T_1 and T_2 generations. *OsTOGR1* expression in Chinese cabbage plants significantly improved the growth performance under high-temperature stress compared to wild-type plants. Moreover, endogenous heat stress-responsive genes of Chinese cabbage such as *NAC069*, *HSP70* and *HSP27B* were also highly up-regulated in transgenic plants under heat stress conditions. These findings clearly proved that *TOGR1* is a good candidate for improving thermotolerance in vegetable crops.

Materials and methods

Plant material

The non-heading Chinese cabbage seeds (*Brassica rapa* ssp. *Chinensis* var. *utilis*, cv. '49Caixin') were kindly provided by Dr. Liu Fan (Beijing Vegetable Research Centre, Beijing, China). The seeds were sown in pots containing nutrient soil (3:1(soil and vermiculate)) under greenhouse conditions (22 °C; 16-h light/8-h dark). One-month-old greenhouse grown plants with few open flowers were used for *in planta* transformation experiments.

Construction of *OsTOGR1* over-expression vector

The over-expression vector *pCAMBIA1300-pHSP:OsTOGR1* was constructed that contains rice *TOGR1* (1.4 kb) driven by a promoter of small heat shock protein 17.9 (*Oshsp17.9*) (1.9 kb) and selectable marker *hpt* gene (1.026 kb) under the control of CaMV 35S promoter. The rice small heat shock protein (*Oshsp17.9*) promoter was amplified from the BAC clone (OSJNBa0079C18) and cloned using *KpnI* and *XbaI* sites of *pCAMBIA1300* (Fig. 1). The rice *TOGR1* gene was cloned downstream of *hsp* promoter using *XbaI* and *HindIII*. The constructed expression vector was mobilized into the *Agrobacterium tumefaciens* strain EHA105 and used for *in planta* transformation of non-heading Chinese cabbage plants.

B.rapa transformation by vacuum infiltration

Greenhouse grown 1-month-old plants (50–60 cm high) with a few open flowers were carefully uprooted from the soil, washed with tap water, and then the total inflorescences were vacuum infiltrated with *A. tumefaciens* strain EHA105 harboring the recombinant plasmid (*pCAMBIA1300-pHSP:OsTOGR1*). The bacterial culture of EHA105 was grown in liquid LB medium at 28 °C for 24–72 h to reach the final concentration of OD600 = 1.0. After centrifugation, the bacterial pellet was resuspended in IM medium (Qing et al. 2000) at two times of the initial culture volume. Three separate groups of 50 number of washed Chinese cabbage plants (above soil portions) were immersed in *Agrobacterium*-containing IM medium (4 L) in a glass vacuum chamber (20 L volume). After 25-min treatment under a vacuum (10 kPa) condition, cabbage plants (150) were transplanted to nutrient soil under greenhouse conditions. These vacuum infiltrated plants were fully covered with polythene bags for a week to prevent water loss and letting them for proper rooting and recovering. Eventually, flowers of infiltrated plants were manually pollinated in green house. After 2 months of pollination, around twenty seeds from each T_0 plant were harvested and germinated on MS medium (Murashige and Skoog 1962) supplemented with the hygromycin (217.14 μM/L) for recovering the putative transformants. The three lines (Br-TOGR1-1, Br-TOGR1-2 and Br-TOGR1-3) of hygromycin resistant plantlets with 1 to 2 leaves were selected and grown in greenhouse. Homozygous lines were generated up to two generations (T_2) by self-pollination, which were used for further experiments.

Molecular analysis of transgenic plants

PCR and RT-PCR analyses

Total genomic DNA was isolated from young leaves of T_1 transgenic (Br-TOGR1-1, Br-TOGR1-2 and Br-TOGR1-3) and wild-type (Br-WT) plants using CTAB method. The 1.0 kb coding region of *TOGR1* and 750 bp coding region of *hpt* genes in T_1 transformants were amplified using gene-specific primer pairs. The PCR cycles were carried out with initial denaturation at 94 °C for 30 s, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 56 °C for 40 s, extension at 72 °C for 60 s and final extension at 72 °C for 5 min. Then the PCR products were electrophoresed on a 1% agarose gel. The young leaves from T_2 transgenic (Br-TOGR1-1, Br-TOGR1-2 and Br-TOGR1-3) and wild-type plants (Br-WT) were used for RNA extraction using TRIzol reagent. The first strand cDNA synthesis kit was used for cDNA synthesis by using DNase-treated total RNA samples (2 μg). The RT-PCR was carried out to check the expression of the *TOGR1* gene in T_2 transgenic plants and *Br-Actin* was used as a loading control. The amplified products were electrophoresed on 1.0% agarose gel. The primers used for the PCR, RT-PCR and q-RT-PCR studies are listed in Table.1

Expression analysis by Real Time RT-PCR

The wild-type (Br-Wt) and T_2 transgenic Chinese cabbage plants grown in greenhouse were transferred to temperature-controlled growth chambers to expose different temperatures

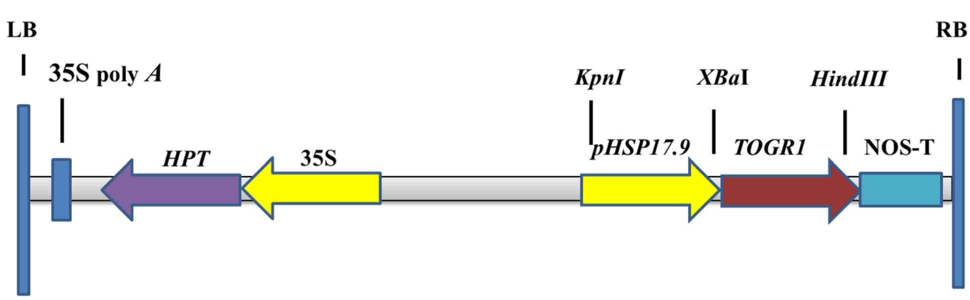

Fig. 1 Schematic representation of *pCAMBIA1300-pHSP:OsTOGR1* expression vector. LB, Left border; RB, Right border; hygromycin phosphotransferase gene (*hpt*) under the control of Cauliflower mosaic virus 35S promoter; thermotolerant growth required 1 (*TOGR1*) gene under the control of small heat shock protein 17.9 promoter (*sHSP17.9*)

Table 1 List of primers for PCR, RT-PCR and qRT-PCR used in this study

Gene	Primer sequence(5′−3′)	Purpose
TOGR1	F: GTGGAGGAGTTGGATGAGGA R: CACATCGGTGCAAATAAGGA	PCR
hpt	F: AATGAGTTGGACCAGCAGAAG R: CATTCAGGTCAAACATAGGCC	PCR
TOGR1	F: CTGCAGCGTGCTTGTCTAAG R: CCCACCCGATGAACATAATC	RT-PCR
BrActin	F: GCTGTTTTCCCCAGTGTTGT R: ACCCTCGTAGATTGGCACAG	RT-PCR
TOGR1	F: TGTCCGGACCTGTGAATCAA R: ACCTGTTTAAGGCGCCTAGT	q-RT-PCR
Bra027596(NAC069)	F: GGCTCGTTACCGATGCGATTAG R: TTGTCGCCTTCTTCGTGGATTC	q-RT-PCR
Bra034104(HSP70)	F: GCCCTCCGTGATGACAAGATAG R: TCTGCTTCAGCCAACTGGTTAC	q-RT-PCR
Bra030036 (HSPB27)	F: ACTAAGAACATGAGCCGTGAGG R: CCTGAGCCAATCGACCAAGAG	q-RT-PCR
BrActin	F: CTCAGTCCAAAAGAGGTATTCT R: GTAGAATGTGTGATGCCAGATC	q-RT-PCR

(22 °C/0 h(before transfer); 22 °C/1 h; 38 °C/1 h; 46 °C/1 h). After temperature stress treatments, the total RNA from leaves was extracted with TRIzol reagent and reverse transcribed with M-MLV Reverse Transcriptase RNaseH. The first-strand cDNA was synthesized using 3 µg of total RNA and oligo (dT) primers in a 20 µL reaction following manufacturer's instructions. The *OsTOGR1* and *Brassica rapa* heat stress-responsive gene (*NAC069*, *HSP70* and *HSP 27B*)-specific primers were designed. All the reaction steps were carried out in a qRT-PCR detection system using SYBR Green supermix. Each experiment was conducted with three biological replicates, and each sample had three technical replicates. The *Br-Actin* gene was used as an internal control to calibrate the relative expression levels of *TOGR1*, *NAC069*, *HSP70* and *HSP 27B* genes in all three T_2 transgenic lines. All the primers used for the real time PCR are listed in Table 1.

Heat shock treatment and evaluation of seedling germination

Wild-type (Br-WT) and T_2 transgenic seeds (Br-TOGR1-1, Br-TOGR1-2 and Br-TOGR1-3) were thoroughly surface sterilized and sown on MS medium. Half portion of the Petri-plate was inoculated with wild-type seeds and remaining half of the plate inoculated with the transgenic seeds. These Petri plates were exposed to heat shock treatment (46 °C) for 1 h. Then the seeds were allowed to germinate and cultivate at normal growth temperature conditions of Chinese cabbage, i.e., 22 °C (16 h light/8 h dark) for 11 days. Germination of wild-type and transgenic plants was observed after 11 days of incubation at 22 °C. The recovered phenotypes from the heat stress of Br-WT and Br-TOGR1-1, Br-TOGR1-2 and Br-TOGR1-3 seedlings were observed, and the survival percentages were enumerated (data not shown).

Heat stress evaluation of transgenic plants

Measurement of hypocotyl length

The transgenic Chinese cabbage (Br-TOGR1-1; Br-TOGR1-2 and Br-TOGR1-3) and Br-WT plant seeds (10 from each) were surface-sterilized and sown on solid MS medium. Then the cultures were incubated for germination at 22 °C. After one day, 90% of the both transgenic and WT plants were germinated and then transferred to culture tubes filled with 1 mL of double distilled water. All these tubes were incubated for 1 h in a water bath at varying temperatures such as 22 °C (optimum), 38 °C (high) or 46 °C (heat), separately. After exposure to different temperatures, the seeds were carefully transferred to petri dishes containing MS basal medium under sterile conditions. Subsequently, the hypocotyl lengths of Br-TOGR1-1; Br-TOGR1-2 and Br-TOGR1-3 and Br-WT were measured after 5 days of culture period at 22 °C.

Measurement of relative electrical conductivity (REC) of leaves

The leaf segments (1 cm) of three independent transgenic plants (Br-TOGR1-1; Br-TOGR1-2 and Br-TOGR1-3) and wild-type plants were harvested before and after temperature treatments (22 °C/1 h, 38 °C/1 h, or 46 °C/1 h). Relative leaf electrical conductivity of transgenic Chinese cabbage plants and wild-type plants treated at 22 °C/1 h, 38 °C/1 h, or 46 °C/1 h was measured as previously described (Jiang et al. 2018). Each designated temperature itself had three replicates for experimental reliability. We calculated the leaf relative electrical conductivity by using the formula, i.e., R3 = (R1/R2) × 100%. Each of the experimental samples had three biological replicates.

Determination of chlorophyll content

The leaf chlorophyll content of the wild-type(Br-WT) and three T_2 transgenic lines (Br-TOGR1-1; Br-TOGR1-2 and Br-TOGR1-3) was determined as described by Liu et al. (2016). Approximately 100 mg of leaf samples were collected from wild-type and three T_2 transgenic lines before and after temperature stress treatment (22 °C/1 h, 38 °C/1 h, or 46 °C/1 h). The collected leaf samples were immersed in 10 ml dimethyl sulfoxide and incubated in dark for 2 days. The absorbance of the solution was read at 645 and 663 nm. Leaf chlorophyll content was calculated by using the formula (0.0127 × OD663 − 0.00269 × OD645) + (0.0029 × OD 645 − 0.00468 × OD663)] × total extract volume/fresh weight of sample.

Statistical analysis

All data were presented as mean values ± SD of three experiments with three replicates. ANOVA method was used to analyze the significance of data differences and *P* value as $P \leq 0.05$. The ΔΔCq method was used for real-time PCR analysis.

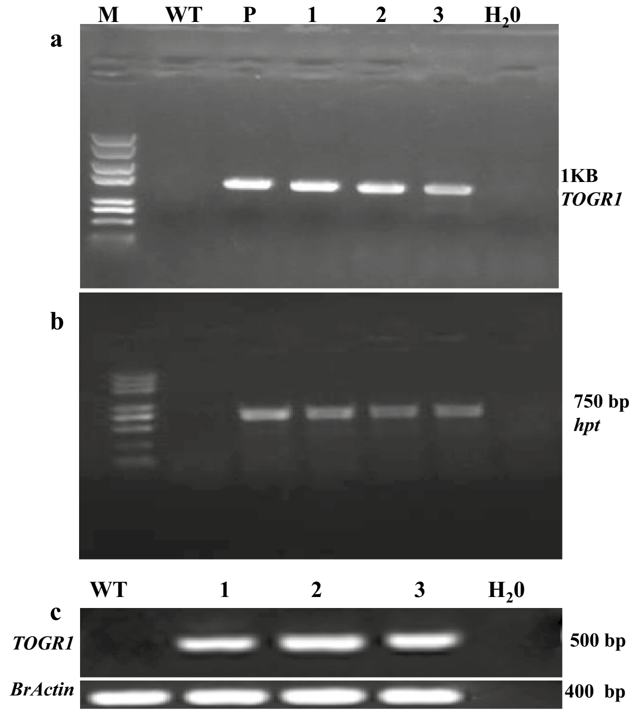

Fig. 2 Molecular analyses of transgenic Chinese cabbage plants. **a** PCR amplification of 1.0 kb fragment of *TOGR1* gene in transgenic plants (T_1). **b** PCR amplification of 750 bp fragment of *hpt* gene in transgenic plants (T_1). **c** RT-PCR expression analysis of *TOGR1* gene in T_2 transgenic plants. *Br-Actin* as a loading control. Lane *M* DNA Marker, *WT* wild-type plant, *P* positive control; 1–3 lanes, transgenic plants of Br-TOGR1-1, Br-TOGR1-2, Br-TOGR1-3; H_2O as a negative control

Results

TOGR1 gene is overexpressed in transgenic *B.rapa* plants

We introduced *pHSP17.9::OsTOGR1* plasmid into the genome of non-heading Chinese cabbage plants via *Agrobacterium* floral infiltration method. The T_0 generation seeds of transgenic plants were harvested and three homozygous transgenic plants (Br-TOGR1-1; Br-TOGR1-2 and Br-TOGR1-3) were screened on hygromycin selection up to two generations (Fig. 4a). Hygromycin-resistant transgenic lines were successfully analyzed by PCR, RT-PCR and real-time PCR analyses for the integration and expression of *TOGR1* gene. Integration of *TOGR1* gene (Fig. 2a) and *hpt* gene (Fig. 2b) in cabbage genome was detected in T_1 generation of transgenic plants via PCR analysis using gene-specific primers. The expression of *OsTOGR1* gene in the three independent transgenic lines (Br-TOGR1-1; Br-TOGR1-2 and Br-TOGR1-3) was also further confirmed by semiquantitative RT-PCR in T_2 generation (Fig. 2c). *Br-Actin* gene was used as a control (Fig. 2c). The relative expression level of the *TOGR1* gene in three independent transgenic and wild-type Chinese cabbage lines was checked by q-RTPCR before (22 °C/0 h) and after different temperature stress treatments (22 °C/1 h, 38 °C/1 h and 46 °C/1 h) (Fig. 3a).The real-time PCR analysis revealed the up-regulation of *TOGR1* gene in leaves of T_2transgenic plants when subjected to temperature treatment at 38 °C (more than 20-fold increase) or 46 °C (more than tenfold increase) compared to 22 °C (fourfold increase) (Fig. 3a). *TOGR1* gene was unexpressed in wild-type plants (Fig. 3a). These results clearly demonstrated that the integration and expression of *OsTOGR1* gene in *B.rapa* genome.

Over-expression of *OsTOGR1* altered the expression levels of some heat stress responsive genes

The heat stress responsive gene expression analysis could provide the partial information for the heat stress tolerance in transgenic plants. Further, we also performed the RT-qPCR to examine the expression of three *B. rapa* spp. *Chinensis* heat stress-responsive genes (*NAC069, HSP70 and HSP 27B*) (Wang et al. 2016b) in three T_2 transgenic

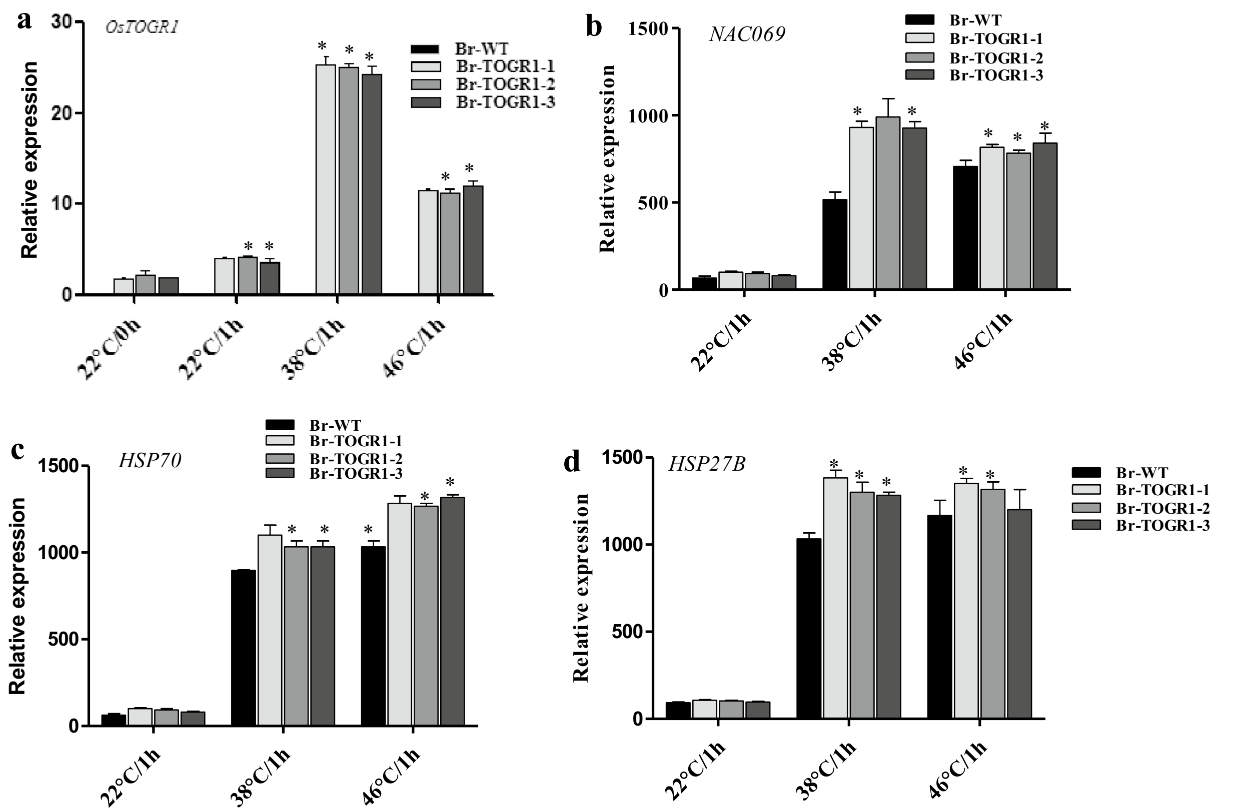

Fig. 3 q-RTPCR analysis of *TOGR1* and heat stress-responsive genes in wild-type and T_2 homozygous transgenic plants (Br-TOGR1-1, Br-TOGR1-2, Br-TOGR1-3) at different temperatures. Expression analysis of **a** *TOGR1* **b** *NACO69* **c** *HSP70* **d** *HSP27b* in leaves of transgenic and wild-type plants after exposure to different temperatures (22 °C or 38 °C or 46 °C/1 h)

lines (Br-TOGR1-1; Br-TOGR1-2 & Br-TOGR1-3) and wild-type plants (Br-WT). Interestingly, the expression levels of *NAC069, HSP70 and HSP27B* were significantly up-regulated in all T_2 transgenic lines compared to wild-type after subjected to high-temperature treatment (38 °C/1 h) or heat stress (46 °C/1 h) (Fig. 3b–d), suggesting that *TOGR1* acts as a positive regulator in inducing other heat stress-responsive genes. The expression level of *TOGR1* was significantly higher in all transgenic plants at both 38 °C (20-fold increase) and 46 °C treatments (tenfold increase) (Fig. 3a) compared to normal temperature treatment (fourfold increase). Similarly, the fold-level expression of heat stress-responsive gene *NAC069* was found to be twofold increase at 38 °C and onefold increase at 46 °C compared to wild-type plants (Fig. 3b). The *HSP70* gene level of expression was found to be 1.5-fold increases at 46 °C and less than onefold increase at 38 °C compared to wild-type plants (Fig. 3c). The *HSP27B* fold-level expression was found to be 1.5-fold increase and 1.2-fold increase compared to wild-type plants (Fig. 3d). These results clearly demonstrated that the expression of *NAC069* and *HSP27B* is significantly higher at 38 °C compared to 46 °C, while *HSP70* expression is high at 46 °C than 38 °C (Fig. 3b–d). To sum up, these results clearly demonstrated that *TOGR1* expression positively co-relates with the expression of these three endogenous heat stress-responsive genes to cope up with the heat stress tolerance in Chinese cabbage plants.

Expression of *TOGR1* confers heat stress tolerance in transgenic cabbage seedlings

To investigate the functional role of *TOGR1* in heat stress tolerance, we have evaluated the heat stress tolerance of transgenic and wild-type plants at seedling stage. The transgenic seeds of T_2 generation and wild-type plants were sown on MS medium and exposed to 46 °C for 1 h. After 1 h of heat shock treatment, they were allowed to germinate at 22 °C for 11 days. After 11 days of incubation, the seeds of Br-TOGR1-1, Br-TOGR1-2 and Br-TOGR1-3 were germinated and showed healthy growth with 2–3 leaves, while the germination of wild-type seeds was arrested (Fig. 4b). These results prompted us that *TOGR1* expression imparts heat stress tolerance for the survival of transgenic seedlings. Moreover, survival rate of transgenic seedlings is

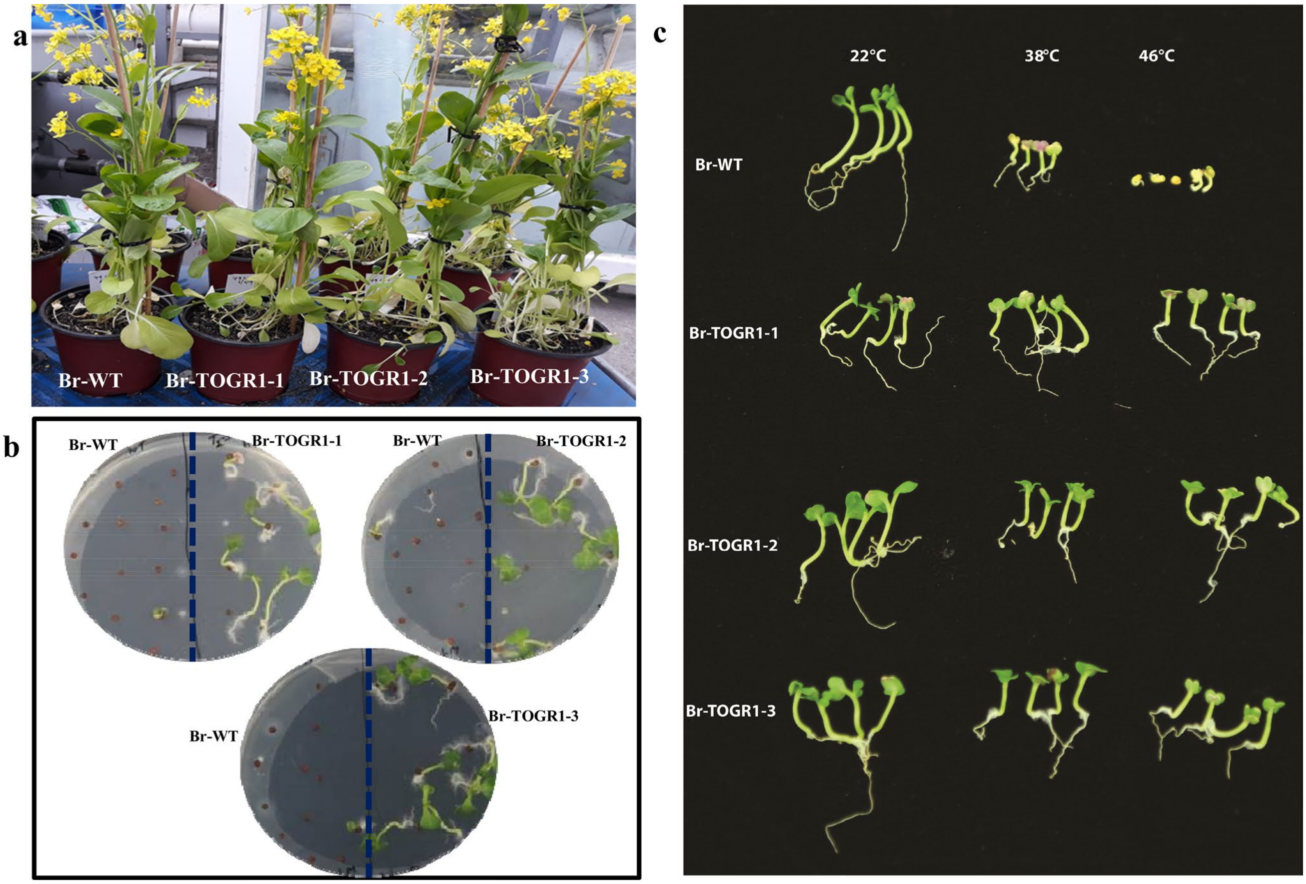

Fig. 4 Phenotypic appearance and heat stress evaluation of transgenic Chinese cabbage plants. **a** Phenotypic appearance of wild type (Br-WT) and T_2 transgenic plants (Br-TOGR1-1, Br-TOGR1-2, Br-TOGR1-3) under normal growth conditions. **b** Seed germination evaluation assay of T_2 transgenic plants (Br-TOGR1-1, Br-TOGR1-2, Br-TOGR1-3) and wild-type plants subjected to 46 °C for 1 h and recovered at 22 °C. **c** Hypocotyls of transgenic and wild-type plants at normal (22 °C), high temperature (38 °C) and heat shock treatment (46 °C)

significantly higher than the wild-type plants (data not shown). These data clearly suggested us that *TOGR1* expressing lines cope up with the heat stress at 46 °C while the wild-type did not. *TOGR1* gene acts like a positive regulator for imparting thermotolerance in transgenic Chinese cabbage plants.

TOGR1 expression increases the hypocotyl length of transgenic cabbage seedlings subjected to high temperature

The hypocotyl length of the transgenic cabbage plants and wild-type plants was measured after 5 days of high temperature (38 °C) and heat shock treatment (46 °C). The germinated transgenic seeds of TOGR1-1, TOGR1-2 and TOGR1-3 were subjected to 22 °C, 38 °C and 46 °C for 1 h. The temperature-treated germinated seeds were then inoculated on MS basal medium and grown at 22 °C for 5 days. After 5 days, we measured the hypocotyl lengths of the transgenic and wild-type seedlings. We found that the hypocotyl lengths of TOGR1-1; TOGR1-2 and TOGR1-3 plants were considerably higher compared to wild-type cabbage plants after high temperature and heat shock treatment (Fig. 4c). Under normal growth conditions, the hypocotyl lengths of wild-type and transgenic plants have similar length (~5 cm) (Figs. 4c and 5b). After subjected to 38 °C for 1 h, the hypocotyl length of all transgenic seedlings attained more than 3 cm and it was less than 2 cm for wild-type plants (Figs. 4c and 5b). After 46 °C treatment for 1 h, the wild-type plants exhibited less than 0.4 cm of hypocotyl length, while the all transgenic plants displayed more than 2 cm of hypocotyl length (Figs. 4c and 5b). This implies that temperature increases result in the suppression of hypocotyl length; however, *TOGR1* expression safe guards the hypocotyl length under heat stress conditions.

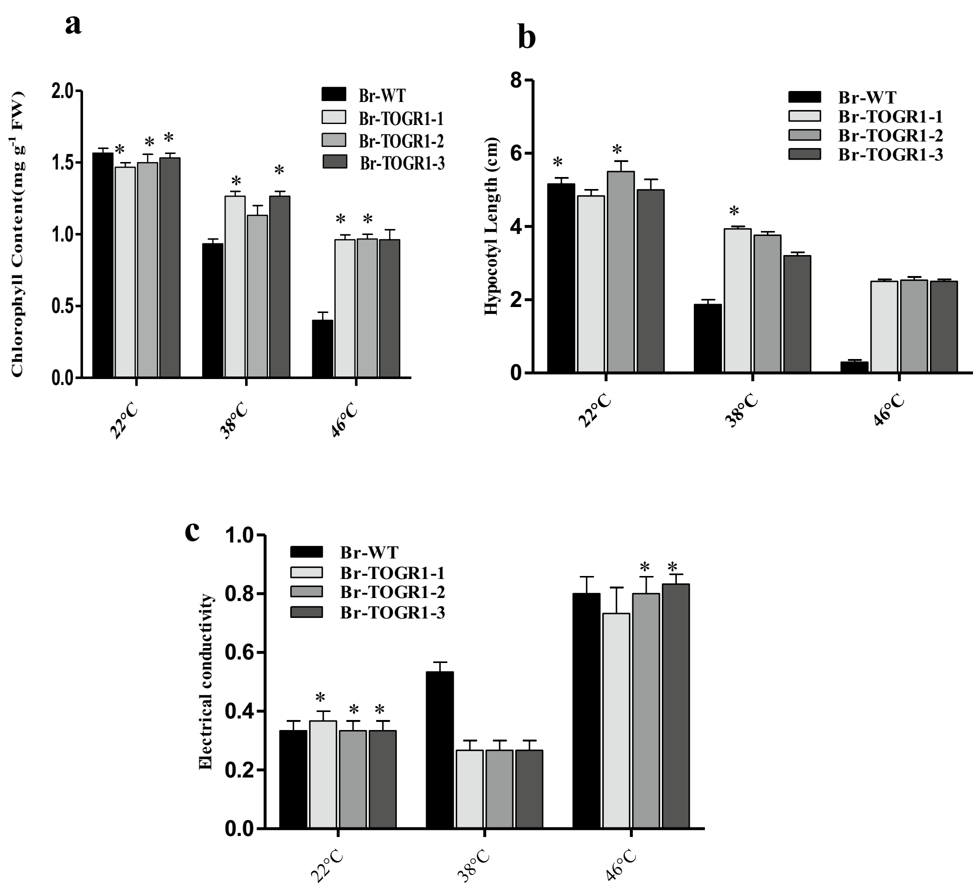

Fig. 5 Chlorophyll content, hypocotyl length and electrical conductivity of wild-type (Br-WT) and T_2 transgenic plants (Br-TOGR1-1, Br-TOGR1-2, Br-TOGR1-3). **a** Chlorophyll content after exposure to 22 °C or 38 °C or 46 °C/1 h. **b** Hypocotyl length after exposure to 22 °C or 38 °C or 46 °C/1 h. **c** Electrical conductivity after exposure to 22 °C or 38 °C or 46 °C. Values are mean ± SD ($n = 3$). Asterisks represent significant difference compared with wild-type at $P \leq 0.05$

Effect of *TOGR1* expression on leaf electrical conductivity under high-temperature and heat shock conditions

Relative leaf electrical conductivity (REC) of three independent T_2 transgenic and wild-type Chinese cabbage plants was measured to enumerate the thermotolerance ability of *pHSP::TOGR1* plants. Under normal growth temperature (22 °C), no obvious changes in REC values were detected in transgenic and wild-type plants. Interestingly, the relative leaf electrical conductivity values of TOGR1-1; TOGR1-2 and TOGR1-3 were reduced significantly relative to those of WT at 38 °C whereas these values not decreased at 46 °C (Fig. 5c).The REC values of transgenic plants after 38 °C/1 h were found to be 0.2, whereas REC values were more than 0.5 for wild-type plants (Fig. 5c). However, the REC values of transgenic plants after treatment at 46 °C/1 h were found to be more than 0.7, whereas those values were more than 0.7 for wild-type plants also (Fig. 5c). Taken together, all these observations clearly demonstrated that the overexpression *TOGR1* in transgenic cabbage seedlings reduces relative leaf electrical conductivity when subjected to high temperature (38 °C) rather than at heat shock treatment (46 °C).

Detection of chlorophyll content in transgenic and WT plants under heat stress

To explore further role of *TOGR1* expression, we determine the chlorophyll content in three transgenic and wild-type plants before and after different temperature treatments. Under high temperature (38 °C/1 h) or heat stress treatments (46 °C/1 h), leaves of three transgenic plants showed significantly higher chlorophyll content than WT plants, but no changes were observed at normal conditions (22 °C). The chlorophyll content of all transgenic lines displayed more than twofold and threefold level compared to wild-type plants under high temperature and heat stress conditions, respectively (Fig. 5a). These results confirmed that the expression of *TOGR1* in Chinese cabbage plants improved the physiological status without diminishing the chlorophyll content under heat stress conditions.

Discussion

Heat stress is one of the major abiotic stresses negatively effects the crop plants, and limiting the crop yield and quality (Lamaoui et al. 2018). Heat stress causes adverse effects on vegetable crop yield and quality (Bisbis et al. 2019). It is an urgent need to develop new and better heat-resilient crop varieties to feed the whole world in changing climate conditions (Singh et al. 2019; Lesk et al. 2016). In general, when plants exposed to heat stress, an array of signaling cascades, metabolite production and expressions of heat stress-associated genes are activated. Understanding the plant heat stress responses at physiological, biochemical, molecular level will facilitate to develop heat stress-tolerant crop varieties (Fahad et al. 2017). Heat stress tolerance in transgenic plants has been achieved by over-expressing heat stress-responsive genes (Grover et al. 2013). The genes involved in heat stress tolerance mechanisms are the best candidates for the development of transgenic vegetable plants that are tolerant to heat stress. In this study, we expressed the *TOGR1* gene from rice in Chinese cabbage plants in response to high-temperature stress. Our results clearly stated that *TOGR1* expressing Chinese cabbage plants performed better growth at seedling stage under heat stress (46 °C) compared to wild-type plants, indicating the genetic engineering is the appropriate approach for overexpressing the heat stress-responsive genes.

Various stress-responsive RNA helicases are previously reported in crops plants. Among them, DEAD-Box RNA helicases are considered to be efficient tools for engineering abiotic stress tolerance in plants (Nidumukkala et al. 2019; Singha et al. 2017; Shivakumara et al. 2017; Augustine et al. 2015). Our previous results showed that over expression of DEAD-Box RNA helicase (*OsTOGR1*) improves rice plant growth and yield under hot conditions (Wang et al. 2016). Our current study further demonstrates that heterologous expression of *TOGR1* in Chinese cabbage plants significantly increases heat stress tolerance associated with improved plant growth, hypocotyl length, relative electrical conductivity, chlorophyll content and up-regulation of few heat stress-responsive genes expression particularly under heat stress conditions (46 °C), compared to wild-type plants.

Analyzing the heat stress tolerance at seedling stage is one of the best methods to evaluate the heat stress tolerance in plants, owing to easiness for analyzing the large number of plants (Lin et al. 2018; Silva-Correia et al. 2014). Compared to wild-type plants, transgenic Chinese cabbage plants (T_2) showed enhanced heat stress tolerance at seedling stage. After subjected to heat stress (46 °C), wild-type Chinese cabbage seeds failed to germinate at normal growth conditions (22 °C), whereas transgenic seeds started germination and developed 2–3 leaves with better survival rate after 11 days of incubation. These results strongly supported that DEAD-Box RNA helicase *TOGR1* acts as a positive regulator for the improved heat stress tolerance in transgenic Chinese cabbage plants. Our results are consistent with the other reports describing the role of DEAD-Box RNA helicases in improving the abiotic stress tolerance in various transgenic crops (Nidumukkala et al. 2019; Singha et al. 2017; Shivakumara et al. 2017; Augustine et al. 2015). However, those reports are strictly concerned with the transgenic plants that are tolerant against salinity, cold, drought stresses. First time, our study revealed the role of DEAD-Box RNA helicase in imparting heat stress tolerance in transgenic plants.

Thermotolerance of Brassica crops under high temperature can be assessed by measuring the hypocotyl elongation (Jiang et al. 2018). Interestingly, hypocotyl length of Br-TOGRR1-1, Br-TOGRR1-2, Br-TOGRR1-3 transgenic seedling was significantly increased at 38 °C and 46 °C compared to Br-WT plants. Whereas electrical conductivity of transgenic plants decreases at 38 °C but not at 46 °C temperature. It has been well established that electrical conductivity is a physiological indicator for heat stress response rather than thermotolerance of plants (Jiang et al. 2018). These data clearly demonstrated the role of *TOGR1* in attributing various morpho-physiological changes to mitigate the heat stress effect in transgenic Chinese cabbage plants. The obtained results are also consistent with previous reports published on morpho-physiological changes in transgenic plants under heat stress conditions (Jiang et al.2018).

Exposure to heat stress causes changes in photosynthesis, leads to retardation in growth and diminishes the crop productivity (Bita and Gerats 2013; Barnabás et al. 2008). Decrease in chlorophyll content is one of the heat stress-induced damage of the plants (Hasanuzzaman et al. 2013). The chlorophyll content of the *TOGR1*-expressing Chinese cabbage plants was significantly higher than the wild-type plants under heat stress conditions, suggesting that DEAD-Box RNA helicases also plays crucial role in protecting photosynthetic apparatus under heat stress by imparting heat stress tolerance in transgenic plants. The over-expression of *TOGR1* in transgenic Chinese cabbage plants may assist in effective rRNA biogenesis needed for primary metabolic processes such as photosynthetic metabolism to enhance the chlorophyll content as well as protection of photosynthetic machinery under heat stress conditions. Previously we reported that *TOGR1* was actively involved in adaptation of primary metabolism under high-temperature conditions (Wang et al. 2016). Gene ontology (GO) enrichment analysis of differentially expressed genes (DEG) in *togr1* mutant rice plants showed severe impairments in enrichment of carbon fixation processes, compared to wild-type plants under high temperature (Wang et al. 2016). Therefore, over-expression of *TOGR1* in Chinese cabbage plants improved the carbon fixation process, resulted in maintaining higher chlorophyll content in transgenic plants under high temperature. These results suggest that *TOGR1* may inhibit the photo-oxidation of chlorophyll under high-temperature conditions.

To explore the effects of *TOGR1* on heat stress responsive genes in transgenic plants, we selected three heat stress responsive genes of *Brassica rapa*spp.chinensis

(*NAC069*, *HSP70* and *HSP 27B*) (Wang et al. 2016b) and examined the expression levels in three transgenic plants along with wild-type plants using q-RTPCR. These heat stress-responsive genes expression levels are up-regulated at 38 °C or 46 °C in transgenic plants, indicating the role of DEAD-Box-RNA helicases in inducing the other abiotic stress-related genes in stress conditions. Many studies reported the simultaneous expression of stress-related genes in transgenic plants expressing DEAD-Box RNA helicase (Singha et al. 2017; Augustine et al. 2015; Zhu et al. 2015). *TOGR1* acts as a thermosensitive RNA chaperone in the nucleolus to protect the plant growth at high temperatures and also may trigger other HSPs/chaperons pathways under high-temperature conditions. These combined chaperone networks promote efficient protein homeostasis under sudden environmental changes. To protect pre-RNA processing during rRNA biogenesis under high temperature, *TOGR1* may coordinate with other HSPs/chaperons in nucleolus. Thus we predict that *TOGR1* may dynamically interconnect with chaperone network to modulate plant growth under high temperature. However, several molecular studies are needed to dissect the link between *TOGR1* and other HSP/chaperon networks. Thus, our results infer that heat stress can induce the expression of *TOGR1* associated with other heat stress-responsive genes in Chinese cabbage plants for heat stress tolerance.

In conclusion, our results clearly demonstrated that the nucleolar DEAD-Box RNA helicase *TOGR1* improves heat resistance in Chinese cabbage plants. Our efforts proved that *TOGR1* surges the survival rate of seedlings by stimulating thermotolerance under heat shock treat through increasing hypocotyl length and decreasing electrical conductivity. Our study provides a platform for the genetic manipulation of Chinese cabbage for improving the thermotolerance. Finally, we propose that *TOGR1* gene may promote thermotolerance by triggering important heat stress-responsive genes in *B.rapa*. Consequently, further research on transgenic Chinese cabbage is required before its adoption for commercial crop improvement.

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Author contribution statement YX and RY conceived and designed the study. RY performed the experiments and analyzed the data. YX and RY drafted the manuscript. All authors read and approved the final manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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